

Nocturnal asthma is associated with reduced glucocorticoid receptor binding affinity and decreased steroid responsiveness at night

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Background: The mechanisms for heightened nocturnal inflammation in patients with nocturnal asthma (NA) are not well understood.

Objective: We sought to determine the glucocorticoid receptor (GR) characteristics and steroid responsiveness in subjects with NA.

Methods: Eleven subjects with NA, 12 subjects with nonnocturnal asthma (NNA), and 16 nonasthmatic control subjects underwent blood sampling at 4 PM and 4 AM in a random order separated by 1 week. GR binding affinity was measured in PBMCs by using a [³H]-dexamethasone (DX) radioligand binding assay and Scatchard analysis. The capacity of hydrocortisone (HC) and DX to suppress proliferation of PBMCs stimulated with PHA was also determined.

Results: The subjects with NA exhibited a significantly lower GR binding affinity at 4 AM, detected by an elevated dissociation constant (K_d) of 22.2 ± 1.6 nmol/L compared with K_d at 4 PM (10.9 ± 0.7 nmol/L; $P = .0001$). The GR K_d of the NNA and control groups did not change significantly from 4 PM to 4 AM. Within the NA group, there was also a significant inverse correlation between the absolute FEV₁ at 4 AM and the K_d at 4 AM ($r = -0.65$, $P = .04$). PBMCs from subjects with NA exhibited less suppression of PBMC proliferation with HC and DX at 4 AM compared with that at 4 PM ($P = .0004$ and $.03$ for HC and DX, respectively). There were no circadian changes in suppression of PBMC proliferation in either the NNA or control groups.

Conclusion: GR binding affinity and steroid responsiveness exhibit a circadian variation in subjects with NA, with a reduced GR binding affinity and suppression of PBMC proliferation at 4 AM that is not observed in normal subjects or asthmatic subjects without nocturnal exacerbation. These observations may contribute to nocturnal airway inflammation by inhibiting the antiinflammatory effects of glucocorticoids. (*J Allergy Clin Immunol* 1999;103:66-71.)

Key words: Glucocorticoid receptors, nocturnal asthma, circadian rhythm

Abbreviations used

DX:	Dexamethasone
GC:	Glucocorticoids
GR:	Glucocorticoid receptor
HC:	Hydrocortisone
K _d :	Dissociation constant
NA:	Nocturnal asthma
NNA:	Nonnocturnal asthma
PEFR:	Peak expiratory flow rate
Rn:	Receptor sites per cell

Nocturnal worsening of pulmonary function is a common and potentially serious complication of asthma because 70% of deaths from asthma occur during the nighttime hours.¹ We have shown that asthmatic subjects who experience nocturnal worsening have greater airway inflammation at 4 AM than at 4 PM, although primarily in the distal alveolar tissue and not the proximal airway tissue.^{2,3} The number of neutrophils, eosinophils and lymphocytes increases in these patients at 4 AM.² The mechanisms for heightened nocturnal inflammation are not well understood. Neurohormonal changes, including cortisol activity and sensitivity to cortisol, are thought to play a role in controlling endogenous allergic tissue inflammation.^{4,5}

One of the mechanisms by which cortisol and other glucocorticoids (GCs) exert their effects is by binding to the glucocorticoid receptor (GR) and translocating to the nucleus where they bind to transcription factor–modulating GC-responsive genes. Previous studies from our group on GC-insensitive asthma have demonstrated an association between GR abnormalities and poor response to steroids.⁶ As a possible mechanism for heightened nocturnal airway inflammation, we hypothesized that GR binding affinity would be reduced at night and is associated with decreased responsiveness to GCs.

METHODS

Subjects

In this prospective study a group of 11 asthmatic subjects with nocturnal asthma (NA), 12 asthmatic subjects without nocturnal worsening, and 16 normal volunteers were recruited from the general Denver community. All asthmatic subjects met diagnostic criteria for asthma,⁷ exhibited a methacholine PC₂₀ of less than 8 mg/mL, and were maintained on short-acting inhaled β_2 -agonist only. The group with nocturnal symptoms demonstrated a fall in peak expiratory flow rate (PEFR) of greater than 15% from bedtime

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TABLE I. Subject characteristics

	NA	NNA	Normal control subjects
Sex	9 M, 2 F	6 M, 6 F	7 M, 9 F
Age (y)*	32.5 ± 6.9	32.3 ± 11.1	28 ± 6.8
Medications (n)	B ₂ -agonist (11), theophylline (1)	B ₂ -agonist (12)	—
4 PM FEV ₁ (%)†	74.9 ± 3.6‡	84.4 ± 3.7‡	97.0 ± 3.1‡
4 AM FEV ₁ (%)	63.3 ± 3.4§	85.2 ± 2.7§	97.6 ± 2.9§
Overnight fall	-21.5 ± 2.8	5.2 ± 2.1†	6.3 ± 2.8†

Overnight fall indicates FEV₁ fall from bedtime to 4 AM.

*Mean ± SD.

†Mean ± SEM.

‡P < .0001 when subjects with NA and NNA were compared with the normal control group; subjects with NA and NNA were not significantly different, P > .05.

§P < .0001, with each group significantly different.

||P < .0001, with each group significantly different.

to morning on at least 4 nights over a 7-day period of testing. The non-nocturnal asthma (NNA) and nonasthmatic groups experienced an overnight fall in PEFr of 10% or less. Exclusion criteria included a history of cigarette smoking, use of cromolyn sodium, use of inhaled or oral corticosteroids, an upper respiratory tract infection within the previous 6 weeks, immunotherapy within the previous 3 months, and/or other medical illnesses. Informed consent was obtained from all patients for this institutional review board–approved protocol.

Protocol

After meeting all inclusion criteria, subjects were scheduled for blood sampling. At 4 PM, 60 mL blood was drawn for the GR assay, and spirometry was performed before and after inhaled β₂-agonist (0.18 mg albuterol). For the 4 AM measurement, subjects arrived at 9:30 PM, spent the night in our NA research center, and were awakened at 4 AM for blood sampling and spirometry. The 4 AM time point was chosen because subjects with NA have demonstrated both increased bronchial reactivity⁸ and airway inflammation that correlates with their decrement in overnight lung function at this time.³

GR binding assay

A [³H]-dexamethasone (DX) radioligand binding assay and Scatchard analysis based on the method of Crabtree et al⁹ and as previously described in our laboratory^{10,11} were used to measure GR binding parameters in PBMCs isolated from blood by Ficoll-Hypaque (Pharmacia LKB Biotechnology, Inc, Piscataway, NJ) gradient centrifugation. After isolation, the PBMCs were washed and incubated in RPMI 1640 (Gibco Labs, Grand Island, NY) for 30 minutes. PBMCs were then exposed to increasing concentrations of [³H]-DX (Amersham Corp, Arlington Heights, Ill) from 0.8 to 400 nmol/L for 60 minutes at 37°C. For measurement of nonsaturable binding, a solution of 20 nmol/L [³H]-DX and 2 μmol/L unlabeled DX was added to the respective tubes. After the latter incubation, 20 μL of supernatant were counted in a β spectrometer to measure free DX concentration. The cell pellet, which measures whole cell DX concentration, was also counted in a β spectrometer to determine the whole cell–associated DX. All values obtained were corrected for nonsaturable binding for each respective concentration. As previously described, saturation binding analysis was performed, assuming a linear binding plot of the bound divided by the free [³H]-DX concentration versus the amount bound and extrapolating to the amount bound at an infinite free-hormone concentration.^{10,11} A least-squares linear regression fit was used to define both receptor sites per cell (Rn) and binding affinity (dissociation constant [Kd]). To assess dynamics of GR binding affinity, a subset of 4 randomly selected subjects from our NA group underwent blood sampling at 4 AM, with GR binding determined 12 hours later at 4 PM.

Finally, reproducibility of the assay for GR Kd has been shown previously.¹¹ When repeated on several asthmatic patients who maintained similar disease activity, the GR binding affinity was found to be highly reproducible, with interclass correlation coefficients of 0.95 for GR number and 0.999 for the GR Kd.

Lymphocyte proliferation assay

Cell proliferation studies were performed at 4 PM and 4 AM. PBMCs were isolated, stimulated with PHA (5 μg/mL) in the presence and absence of hydrocortisone (HC) or DX at concentrations of 10⁻⁵ to 10⁻⁹ mol/L (Sigma Chemical Co, St Louis, Mo), and plated at 2 × 10⁵ cells/200 μL/well in 96-well microtiter plates. After incubation at 37°C and 5% CO₂ for 3 days, [³H]-thymidine (5 μCi/mL) was then added to each well and incubated for 6 hours. The cells were then harvested onto glass fiber filter plates, and [³H]-thymidine incorporation was measured.

Statistical analysis

Overnight fall in FEV₁ was compared across the groups by using ANOVA.¹² Overnight time-response curves for Kd, log₁₀(Rn), and steroid dose-response curves for PBMC proliferation at each time point were compared for all groups by using a mixed-effects model with fixed effects for time, group, and the interaction between time and group, plus a random subject effect.^{13,14} When the interaction was significant, pairwise comparisons of least-squares means were made within groups and within times. Rn was log transformed before analysis to obtain more normally distributed residuals. All tests were 2 sided and conducted at the 5% significance level. Data are expressed as mean ± SEM or SD if normally distributed or median with interquartile range if not normally distributed or as a percentage of positive control. The sample size reflects a power of 80% to detect a difference of 0.5 log in the steroid concentration that produced a 50% reduction in proliferation (IC₅₀) for DX and HC, with a type I error rate of 5%.

RESULTS

Subject characteristics and FEV₁ measurements

The subject characteristics of our 3 groups are shown in Table I. All subjects with NA exhibited a fall in overnight PEFr of greater than 15% in 7 of 7 nights at home before the study, and subjects with NNA and control subjects exhibited an overnight fall in PEFr of less than 10% in 7 of 7 nights at home before the study. Prebronchodilator FEV₁ fell from 3.1 ± 0.2 L at 4 PM to

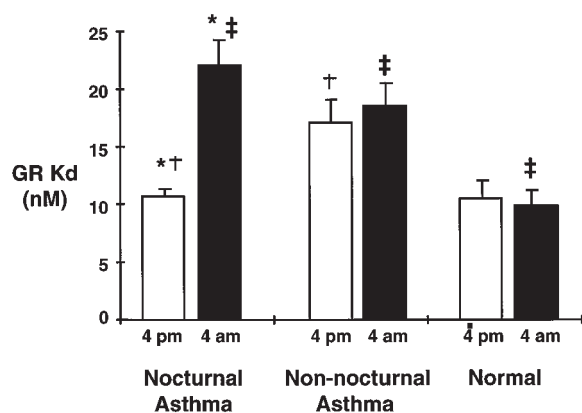


FIG 1. The Kd from 4 PM to 4 AM in the NA, NNA, and normal groups. The higher the Kd, the lower the GR-binding affinity. Open bars represent the Kd at 4 PM, and filled bars represent the Kd at 4 AM. * $P < .0001$ when 4 PM Kd is compared with 4 AM Kd within the NA group; † $P < .0009$ when subjects with NA and NNA are compared at 4 PM; and ‡ $P < .0001$ when asthmatic groups are compared with control group.

2.7 ± 0.2 L at 4 AM in the NA group ($P = .05$). The FEV_1 remained stable at these time points in the NNA group (2.9 ± 0.2 L to 3.1 ± 0.3 L) and in the normal group (4.2 ± 0.2 L to 4.1 ± 0.7 L; $P > .05$). The 4 PM percent predicted FEV_1 was significantly lower in the asthmatic groups compared with the control group ($P < .0001$), but the asthmatic groups were not significantly different from each other ($P > .05$). At 4 AM, the percent predicted FEV_1 was significantly different among the 3 groups, with the subjects with NA exhibiting the lowest percent predicted FEV_1 at $63.3\% \pm 3.4\%$ ($P < .0001$).

GR binding assay

The GR Kd is inversely proportional to the binding affinity. Thus the higher the Kd, the lower the GR binding affinity. There was a significant difference in PBMC GR affinity at different time points only in the NA group (Fig 1). From 4 PM to 4 AM, the GR Kd increased from 10.9 ± 0.7 to 22.2 ± 1.6 nmol/L ($P = .0001$). In contrast, there were no significant changes in the GR binding affinity in the NNA and normal groups at these time points. The GR Kd values in the NNA group were 16.7 ± 1.4 and 18.4 ± 1.9 nmol/L at 4 PM and 4 AM, respectively, and the normal group Kd values were 10.7 ± 0.7 and 10.1 ± 0.7 nmol/L. Interestingly, a significant difference was observed between the GR Kd for both asthmatic groups and normal subjects at 4 AM ($P = .0001$) (Fig 1). Reversibility of GR binding affinity over a 12-hour period is demonstrated in the subset of 4 subjects with NA. The Kd at 4 AM was 26 ± 4.0 nmol/L and decreased to 12.3 ± 6.0 nmol/L at 4 PM ($P = .02$).

Within the NA group, there was also a significant inverse correlation between the absolute FEV_1 at 4 AM and the Kd at 4 AM, suggesting that the higher the Kd at 4 AM, the lower the FEV_1 ($r = -0.65$, $P = .04$). This relationship was not appreciated at 4 PM ($r = 0.1$, $P = .74$).

The $\log_{10}$ of Rn (expressed as number of receptor sites/cell) in the NA group was 3.32 ± 0.05 at 4 PM and 3.60 ± 0.03 at 4 AM ($P = .0006$). The variation from 4 PM to 4 AM did not reach statistical significance in the NNA and normal groups. The values for the NNA group at 4 PM and 4 AM were 3.55 ± 0.05 and 3.56 ± 0.11 , respectively ($P = .95$); the $\log_{10}$ Rn values in the normal group were 3.28 ± 0.04 and 3.28 ± 0.04 ($P = .92$).

Lymphocyte proliferation studies

To determine whether the change in GR binding affinity was functionally important, we examined the ability of HC and DX to suppress lymphocyte proliferation of PBMCs from subjects with NA at 4 AM versus 4 PM. The suppression of proliferation by HC and DX was significantly decreased at 4 AM compared with that at 4 PM, but only within the NA group ($P = .0004$ and $.03$ for HC and DX, respectively) (Figs 2 and 3). However, no change in suppression of proliferation with HC or DX was observed within the NNA and control groups at either time point (NNA: $P = .65$ and $.53$ for HC and DX; control group: $P = .93$ and $.85$ for HC and DX) (Figs 2 and 3).

DISCUSSION

In this study we have shown for the first time that PBMC GR binding affinity has a circadian variation in patients with NA, but not in normal subjects or asthmatic subjects without nocturnal worsening of lung function. In addition, subjects with NA exhibited reduced steroid responsiveness at night as illustrated by the reduced ability of either HC or DX to decrease their lymphocyte proliferation at night compared with daytime. The concentrations of HC and DX used in these studies are clinically relevant as physiologic concentrations are 10^{-7} mol/L and 10^{-8} mol/L, respectively. The GR Kd values of the NA group increased 2-fold from 4 PM through 4 AM, indicating a significant reduction in the binding affinity of these receptors to DX, with significantly lower affinity at 4 AM compared with 4 PM.

A potential critique is whether these changes in Kd are biologically important. Previous reports in patients with primary cortisol resistance have found that a decrease in the 2- to 3-fold range is biologically important.¹⁵⁻¹⁷ Chrousos et al¹⁸ report a father and son who, despite having significantly elevated plasma cortisol levels, displayed none of the stigmata of Cushing's syndrome. In addition, they were "resistant" to adrenal suppression by DX. Analysis of these patients' fibroblast and mononuclear cell GRs revealed diminished binding GR affinity compared with normal subjects, with a dissociation constant (Kd) 2 to 3 times higher than that of control cells. The authors noted that the greater the Kd, the greater the amount of DX required to diminish their morning cortisol level. The authors concluded that the diminished GR binding affinity accounted for the above derangements.

The data provided by this study suggest that GR binding affinity is higher in normal subjects compared with asthmatic subjects at night, which was demonstrated by comparing the Kd of both the NA and NNA group with

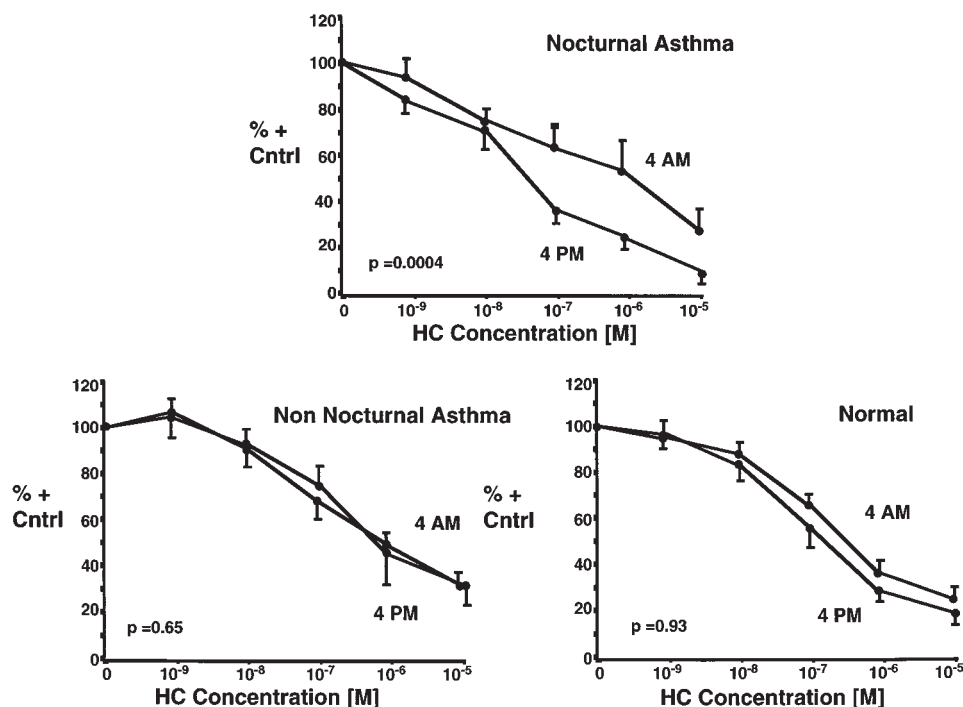


FIG 2. Suppression of proliferation by HC at 4 PM and 4 AM in NA group (*top*), NNA group (*bottom left*), and normal control subjects (*bottom right*) is shown. Values are means $\pm$ SEM.

the K_d of the normal group at 4 AM. The K_d also inversely correlates with the FEV_1 at 4 AM, suggesting that the higher the K_d , the lower the 4 AM FEV_1 ($r = -0.68$, $P = .04$). This relationship was not present during the day, when the K_d at 4 PM did not correlate with the FEV_1 ($r = 0.1$, $P = .74$). These observations suggest that the altered binding affinity may be related to lung function at night, but not during the day.

Decreased GR binding affinity of PBMCs is known to be associated with steroid unresponsiveness. PBMCs from subjects with chronic asthma exhibit a spectrum of GR binding abnormalities. These abnormalities are characterized by poor GR affinity in the majority of patients and are most pronounced in those with poorly controlled asthma.^{6,10,19} The defect in GR affinity associated with steroid unresponsiveness has been shown to be cytokine-induced, restricted to T cells, and detected by measuring PBMC GR-binding characteristics, as we did in this study. The particular cytokines shown to induce GR alterations are the combination of IL-2 and IL-4, as shown by Kam et al.¹⁹ They incubated PBMCs from normal subjects with IL-2 and IL-4 for 48 hours. The binding affinity significantly decreased compared with media alone (K_d : 36.1 ± 1.63 nmol/L vs 6.7 ± 0.5 nmol/L) and was shown to be localized to the T-cell population. The mechanism by which IL-2 and IL-4 induce defects in GR binding is currently unknown.

In subjects with NA we have shown that IL-4 is increased at night, particularly in the lung parenchyma as compared with subjects with NNA.²⁰ The cytokine milieu during the day and night in NA has not been completely

determined, but studies have shown increases in IL-1 and IL-5 at night compared with the day.²⁰⁻²² Although inflammation is present in these patients during the day and night, it may be that significant reduction of particular cytokines that results in the near normalization of GR binding affinity occurs in subjects with NA during the day. Another possibility is that the cytokine profile of subjects with NA may be different from that of subjects with NNA during the day, suggesting that they are different from subjects with NNA. Indeed, reversibility of abnormal GR binding affinity was seen in our subjects with NA in 12 hours as compared with 48 hours in the steroid-insensitive asthmatic subjects,¹⁹ paralleling the change in airway inflammation.

Besides reduced GR binding affinity, reduced binding of the GR to the DNA binding site has been shown in steroid-insensitive asthmatic subjects by Adcock et al.²³ This reduced DNA binding may be secondary to an abnormal interaction of the hormone-receptor complex with the transcription factor activator protein-1.²³ Because the abnormality of GR binding is associated with increased inflammation at night in NA, but not with daytime inflammation, these issues merit consideration as an additional possible mechanism of steroid unresponsiveness in our subjects.

The question frequently arises as to whether patients with NA simply have more severe asthma. In the subjects with NA recruited for our studies, lung function during the day is sometimes reduced or in the normal range.^{2,3} The changes appreciated only at night in subjects with NA, such as increased airway inflammation and decreased steroid responsiveness, can be seen in subjects with severe

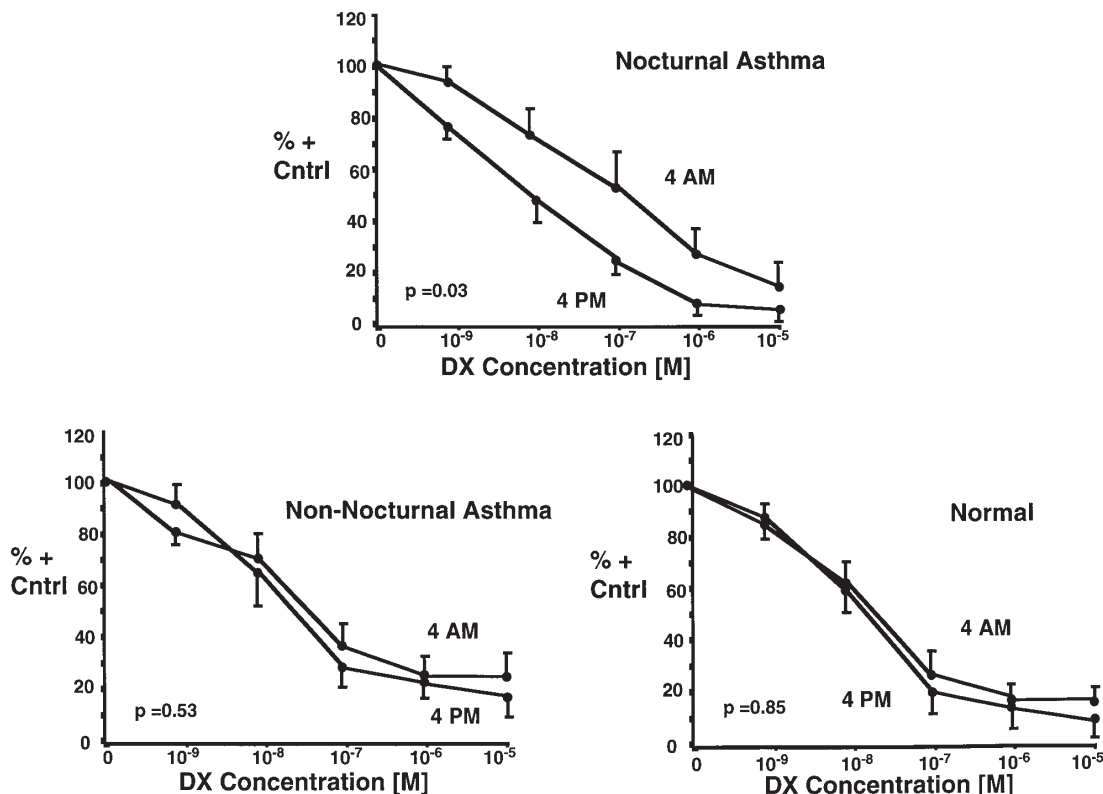


FIG 3. Suppression of proliferation by DX at 4 PM and 4 AM in NA group (top), NNA group (bottom left), and normal control subjects (bottom right) is shown. Values are means $\pm$ SEM.

asthma. However, these immune alterations are observed during the day in this latter group.^{6,24} In addition, Wenzel et al²⁴ compared airway and parenchymal inflammation in subjects with asthma, as compared with a subset of our subjects with NA, and found an increase in alveolar tissue neutrophils in the group with severe asthma. This was not appreciated in the subjects with NA because the eosinophil was the main effector cell. There has also been data to suggest that β_2 -agonists, especially long-acting inhaled β_2 -agonists, exert anti-GC activity by decreasing the action of endogenous GCs.²⁵ In this study both NA and NNA groups were maintained on short-acting inhaled β_2 -agonists only and received similar doses in the laboratory, making this possibility less likely. Thus subjects with NA may have a unique asthmatic inflammatory response.

Recent studies have shown that the magnitude of allergic inflammation responses is modulated by endogenous cortisol secretion. Herrscher et al,⁴ investigating the effect of endogenous cortisol on IgE-dependent cutaneous reactions, demonstrated that decreased levels of serum cortisol are associated with increased allergic inflammation by affecting the expression of cellular events at late-phase sites. In this study we demonstrate that the circadian variation in GR affinity may also contribute to a reduction in the effects of endogenously secreted cortisol from day to night. Reduced GR binding affinity at 4 AM may thus intensify the consequences of the cortisol nadir seen at

night and related, at least in part, to decreased steroid responsiveness and exacerbations of airway inflammation during the night.²⁶ Although serum cortisol levels are not different between the subjects with NA, subjects with NNA, and control subjects overnight,²⁷ the increasing cortisol seen in all subjects at 7 to 8 AM may provide enough antiinflammatory action to decrease IL-2 and IL-4 in the subjects with NA and thus improve binding affinity. Further study is required to prove this hypothesis.

We conclude that an improved knowledge of GR characteristics may be important in the development of new treatments for NA, as well as in the selection of agents, doses, and/or schedules for individual therapies. Further studies are necessary to determine the cell type involved, concomitant alterations in the lung, and the mechanisms causing this interesting phenomenon. The results of our current study demonstrate that GR binding affinity diminishes from 4 PM to 4 AM in patients with NA and that the binding affinity at night is significantly correlated to nighttime lung function. This may contribute to nocturnal airway inflammation by blunting the antiinflammatory effects of GCs at night.

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