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Research Article

Nocturnal asthma represents a unique subset of patients with asthma who experience worsening symptoms and airflow obstruction at night. The basis for this phenotype of asthma is not known, but beta 2-adrenergic receptors (beta 2AR) are known to downregulate overnight in nocturnal asthmatics but not normal subjects or nonnocturnal asthmatics. We have recently delineated three polymorphic loci within the coding block of the beta 2AR which alter amino acids at positions 16, 27, and 164 and impart specific biochemical and pharmacologic phenotypes to the receptor. In site-directed mutagenesis/recombinant expression studies we have found that glycine at position 16 (Gly16) imparts an accelerated agonist-promoted downregulation of beta 2AR as compared to arginine at this position (Arg16). We hypothesized that Gly16 might be overrepresented in nocturnal asthmatics and thus determined the beta 2AR genotypes of two well-defined asthmatic cohorts: 23 nocturnal asthmatics with 34 +/- 2% nocturnal depression of peak expiratory flow rates, and 22 nonnocturnal asthmatics with virtually no such depression (2.3 +/- 0.8%). The frequency of the Gly16 allele was 80.4% in the nocturnal group as compared to 52.2% in the nonnocturnal group, while the Arg16 allele was present in 19.6 and 47.8%, respectively. This overrepresentation of the Gly16 allele in nocturnal asthma was significant at P = 0.007 with an odds ratio of having nocturnal asthma and the Gly16 […]

Genetic Polymorphisms of the β_2-Adrenergic Receptor in Nocturnal and Nonnocturnal Asthma

Evidence that Gly16 Correlates with the Nocturnal Phenotype

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Abstract

Nocturnal asthma represents a unique subset of patients with asthma who experience worsening symptoms and airflow obstruction at night. The basis for this phenotype of asthma is not known, but β_2-adrenergic receptors (β_2AR) are known to downregulate overnight in nocturnal asthmatics but not normal subjects or nonnocturnal asthmatics. We have recently delineated three polymorphic loci within the coding block of the β_2AR which alter amino acids at positions 16, 27, and 164 and impart specific biochemical and pharmacologic phenotypes to the receptor. In site-directed mutagenesis/recombinant expression studies we have found that glycine at position 16 (Gly16) imparts an accelerated agonist-promoted downregulation of β_2AR as compared to arginine at this position (Arg16). We hypothesized that Gly16 might be overrepresented in nocturnal asthmatics and thus determined the β_2AR genotypes of two well-defined asthmatic cohorts: 23 nocturnal asthmatics with 34±2% nocturnal depression of peak expiratory flow rates, and 22 nonnocturnal asthmatics with virtually no such depression (2.3±0.8%). The frequency of the Gly16 allele was 80.4% in the nocturnal group as compared to 52.2% in the nonnocturnal group, while the Arg16 allele was present in 19.6 and 47.8%, respectively. This overrepresentation of the Gly16 allele in nocturnal asthma was significant at $P = 0.007$ with an odds ratio of having nocturnal asthma and the Gly16 polymorphism being 3.8. Comparisons of the two cohorts as to homozygosity for Gly16, homozygosity for Arg16, or heterozygosity were also consistent with segregation of Gly16 with nocturnal asthma. There was no difference in the frequency of polymorphisms at loci 27 (Gln27 or Glu27) and 164 (Thr164 or Ile164) between the two groups.

Thus the Gly16 polymorphism of the β_2AR, which imparts an enhanced downregulation of receptor number, is overrepresented in nocturnal asthma and appears to be an important genetic factor in the expression of this asthmatic phenotype. (*J. Clin. Invest.* 1995. 95:1635–1641.) Key words: downregulation • G-protein • allele-specific polymerase chain reaction

Introduction

Patients with nocturnal asthma represent a subset of asthmatics who experience a marked worsening of airway obstruction and symptoms while asleep (1, 2). Typically, in these patients symptoms and airway obstruction are worse in the early morning hours. In addition to increased airway resistance and decreased peak expiratory flow rate, nocturnal asthmatics display greater bronchial hyperreactivity as compared to nonnocturnal asthmatics during this time (3, 4). While the pathophysiologic basis for nocturnal asthma is not clear, several studies have suggested that autonomic function may be different in nocturnal asthma as compared to nonnocturnal asthma (5–8). Particularly germane to the current study, we have shown in a previous study that circulating neutrophil and lymphocyte β_2-adrenergic receptors (β_2AR),[1] which are potential markers for β_2ARs of bronchial smooth muscle and other lung cells, decrease at 4:00 a.m. compared to 4:00 p.m. in patients with nocturnal asthma (7). No such downregulation of β_2AR was found in nonnocturnal asthmatics or normal subjects. The mechanism responsible for this nocturnal β_2AR downregulation in nocturnal asthma is not known, but given the role of β_2AR in modulating bronchial smooth muscle relaxation and other potentially important functions in asthma, such downregulation may be an important event in the development of nocturnal asthma.

Recently, we have found that there is heterogeneity in the structure of the β_2AR in the human population (9). Six different polymorphic forms of the β_2AR have been identified. These polymorphisms consist of amino acid substitutions at the positions indicated in Table I. We have mimicked these polymorphisms by site-directed mutagenesis of the cloned human β_2AR cDNA, expressed them in Chinese hamster fibroblasts, and found that some of these β_2ARs indeed display different pharmacologic properties (10, 11). Of particular interest in regard to asthma, the presence of glycine at position 16 of the β_2AR imparts enhanced agonist-promoted downregulation (11). Having defined the pharmacologic and biochemical phenotype of this receptor polymorphism in vitro, in the present study we have considered that Gly16 might be responsible for the decrease in β_2AR expression found in patients with nocturnal asthma and would be overrepresented in a population of nocturnal patients with this asthmatic phenotype. To pursue this, we

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1. *Abbreviations used in this paper:* β_2AR, β_2-adrenergic receptor; FEV_1, forced expiratory volume in 1 s; PEFR, peak expiratory flow rate.

Table I. Polymorphisms of the Human β_2AR and Their Phenotypes

Nucleic acid	Nucleic acid	Amino acid	Amino Acid	Receptor phenotype
No.		*No.*		
46	A	16	Arg	Reference (wild-type)
	G		Gly	Enhanced downregulation
79	C	27	Gln	Reference (wild-type)
	G		Glu	Absent downregulation, immature form
491	C	164	Thr	Reference (wild-type)
	T		Ile	Altered binding, coupling, sequestration

The wild-type β_2AR, as delineated by the original cloning of the receptor (24), is Arg16, Gln27, Thr164 and is denoted as *reference*. Another polymorphism, consisting of Met substituted for Val at amino acid 34, is present in < 1% of the population and does not change the receptor phenotype. Summarized from references (9–11).

determined the β_2AR genotype in a group of well-defined nocturnal and nonnocturnal asthmatics.

Methods

Patient selection. All subjects met the criteria for asthma as outlined by the Expert Panel Report of the National Asthma Educational Panel (12) and the study was approved by the Institutional Review Boards for Human Studies at both institutions. To distinctly separate the asthmatic groups into those with nocturnal asthma vs those with nonnocturnal asthma, home peak flow monitoring was performed. Peak expiratory flow rates (PEFR) were measured by the subjects using a peak flow meter (Mini-Wright; Armstrong Industries, Northbrook, IL). Nocturnal asthma was defined as five consecutive bedtime to morning peak flow decrements of ≥ 20%. The nonnocturnal asthma group required five consecutive peak flow decrements of ≤ 10%. Consecutive volunteers who met these criteria were enrolled into the study until there were a total of 45 subjects, 23 having nocturnal asthma and 22 having nonnocturnal asthma. 10 patients were eliminated due to lack of peak flow criteria. Baseline (daytime) spirometry was performed (Moose Spirometer; Cybermedic, Louisville, CO) after 10:00 a.m. in the pulmonary function laboratory. Since asthma and the nocturnal worsening of asthma can change over time, the 45 subjects were also categorized in a blinded fashion as being nocturnal or nonnocturnal by history. A positive history was defined as nocturnal awakening due to asthma occurring on an average of at least once a week for 12 consecutive months.

β_2AR genotyping. Personnel performing β_2AR genotyping were blinded as to the assignment of patients to nocturnal and nonnocturnal groups. No patient in either group had been a participant in our previous study (9). Polymorphisms of the β_2AR coding block were delineated using an allele-specific PCR approach (13). Allele-specific PCR is based on the premise that under the appropriate conditions, a match between template and primer at the most 3′ nucleotide is necessary for the generation of a PCR product and that mismatches result in no product. The known polymorphisms of the human β_2AR were previously established in our laboratory by the use of temperature gradient gel electrophoresis as a screening technique followed by direct dideoxy sequencing (9). Allele-specific PCR was used in the current study to provide a more rapid determination of polymorphisms as compared to this latter method. For the current study, allele-specific PCR was performed to assess polymorphisms at nucleic acids 46, 79, and 491, which result in changes in the encoded amino acids at positions 16, 27, and 164 of the receptor protein (Table I). As further discussed below, each of these polymorphisms imparts a unique receptor phenotype (10, 11). The genotypes are abbreviated as Arg16, Gly16, Gln27, Glu27, Thr164, and Ile164. Genomic DNA was isolated from 2 ml of peripheral blood by a cetylmethyl ammonium bromide separation technique (14). PCR reactions were carried out in a vol of 100 μl using ~ 500 ng of genomic DNA. To delineate the two polymorphisms at nucleic acid 46 (amino acid 16), the primer pairs were: 5′-CTTCTTGCTGGCACCCAATA-3′ (sense) and 5′-CCAATTTAGGAGGATGTAAACTTC-3′ (antisense) or the same antisense primer and 5′-CTTCTTGCTGGCACCCAATG-3′ (sense). The generated PCR product size using these primers is 913 bp. Primer pairs for delineating the two polymorphisms at nucleic acid 79 (amino acid 27) were 5′-GGACCACGACGTCACGCAGC-3′ (sense) and 5′-ACAATCCACACCATCAGAAT-3′ (antisense), or the same antisense primer and 5′-GGACCACGACGTCACGCAGG-3′ (sense). Use of these primers results in a product with a molecular size of 442 bp. For detection of the polymorphisms at nucleic acid 491 (amino acid 164) primer pairs were '5-TGGATTGTGTCAGGCCTTAT-3′ (sense) and '5-CACAGCAGTTTATTTTCTTT-3′ (antisense), or the same antisense primer and '5-TGGATTGTGTCAGGCCTTAC-3′ (sense). The PCR product size from these primers is 662 bp. For detection of polymorphisms at nucleic acids 46 and 79, the polymerase Vent exo (-) (New England Biolabs, Inc., Beverly, MA) was used for these reactions. For detection of the polymorphisms at nucleic acid 491, the Stoffel fragment of Thermus aquaticus (*Taq*) polymerase was used. Reaction buffers were those included with these polymerases from the manufacturers. Temperature cycling was 98°C for 30 s, 66–68°C for 45 s, and 72°C for 45 s for 30 cycles. 20 μl of the PCR reactions were then electrophoresed on 1% agarose gels and visualized with ethidium bromide staining and ultraviolet illumination. The allele-specific PCR technique was verified by direct dideoxy sequencing of PCR products generated using sequencing primers different from those used in the PCR. In addition, plasmids consisting of wild-type β_2AR cDNA or mutated cDNA corresponding to the above polymorphisms were used as positive and negative control templates for the allele-specific PCR studies. Methods used to develop these constructs were as previously described (10, 11).

Materials. Vent exo (-) was from New England Biolabs Inc., (Beverly, MA). Taq (Stoffel fragment) and dNTP's were from Perkin-Elmer Cetus Instruments (Norwalk, CT). Oligonucleotide primers were synthesized with an Applied Biosystems, Inc. Oligonucleotide Synthesizer (Foster City, CA). All other reagents were from Sigma Chemical Co. (St. Louis, MO).

Statistical analysis. The relationship between polymorphisms and nocturnal vs nonnocturnal asthma were evaluated with Fisher's Exact Test, with a two-sided P value < 0.05 considered significant. For 3 × 2 contingency tables, where the number of subjects in each group was small, the Chi-squared Test for Trend was used with a P value < 0.05 considered significant. The odds ratio was calculated using the method of Woolf (15). Physiologic variables are shown by the mean±SE and were compared using the Mann–Whitney U test, with a P value of < 0.05 considered significant.

Results

Asthmatic cohorts. Table II shows the demographic data for the asthmatic groups. The nocturnal asthma group consisted of 23 patients (mean age of 33.9±1.3 yr, 14 male and 9 female), and the nonnocturnal asthma group consisted of 22 patients (mean age of 36.8±2.2 yr, 9 male and 13 female). The cohorts were selected for the study by overnight decrements in PEFR. By this criteria the nocturnal asthma group had a mean overnight decrement in PEFR of 34.0±2.0% compared to 2.3±0.8% for the nonnocturnal asthma group (P = 0.0001). The mean daytime baseline percent predicted forced expiratory volume in 1 s (FEV_1) values were 70.9±3.1% vs 85.1±3.0% for the nocturnal and nonnocturnal cohorts, respectively (P = 0.002). As

Table II. Demographic Data for the Nocturnal and Nonnocturnal Asthmatic Cohorts

	Gender	Age	Race	Overnight decrease PEFR	Medication use Iβ_2, T, IS	Predicted FEV_1 daytime
		yr		*%*	*No. of patients*	*%*
Defined by PEFR						
NA (n = 23)	14M:9F	33.9±1.3	20C:3H	34.0±2.0*	22, 9, 8	70.9±3.1‡
NNA (n = 22)	9M:13F	36.8±2.2	17C:3B:2H	2.3±0.8	22, 0, 1	85.1±3.0
Defined by history						
NA (n = 31)	17M:14F	35.3±1.4	26C:2B:3H	—	30, 9, 8	73.5±2.8§
NNA (n = 14)	6M:8F	35.4±2.7	11C:1B:2H	—	14, 0, 1	87.4±3.2

NA, nocturnal asthma; NNA, nonnocturnal asthma; C, Caucasian; B, Black; H, Hispanic; Iβ_2, inhaled β_2-agonist; T, theophylline; IS, inhaled corticosteroids. * P = 0.0001 vs nonnocturnal; ‡ P = 0.002 vs nonnocturnal; § P = 0.004 vs nonnocturnal.

shown in Table II, virtually all patients used inhaled β-agonists on a regular basis. The use of theophylline and inhaled corticosteroids was prominent in the nocturnal asthma group as compared to the nonnocturnal group. No patient in either group was receiving oral corticosteroids or immunotherapy. Table II also shows the demographic data expressed by a history of nocturnal asthma occurring on an average of at least once a week over 12 consecutive months. This historical phenotyping increased the number in the nocturnal asthma group from 23 to 31 and decreased the nonnocturnal asthma group from 22 to 14. The percent predicted FEV_1 (daytime) remained similar to the prior two groups (Table II) and the between group difference was significant (P = 0.004).

Allele-specific PCR. Fig. 1 illustrates the specificity of the allele-specific PCR for delineating polymorphisms at nucleic acid 46 (corresponding to amino acid 16). As shown in Fig. 1 *A*, when reactions were carried out using plasmids containing β_2AR sequences encoding for Arg16 or Gly16 as templates, PCR products resulted only when the 3′ nucleotide of the primers matched with that of the template sequence. When both plasmids were present (mimicking the heterozygous state) products were obtained with both primers. Fig. 1 *B* shows the results of allele-specific PCR from three patients who were either homozygous for Arg16, homozygous for Gly16, or heterozygous at this locus. Dideoxy sequencing, shown in Fig. 1 *C*, confirms the presence of the different polymorphisms at this site. Primer pairs designed for the identification of polymorphisms at nucleic acids 79 and 491 (amino acids 27 and 164) were also tested in the same manner as that described above, and each was capable of specifically detecting the intended polymorphism (data not shown).

β_2AR genotypes of nocturnal and nonnocturnal asthmatics. Allele-specific PCR was performed on the 45 patients from the two cohorts to delineate polymorphisms at amino acids 16, 27, and 164. The results, as shown in Tables III and IV, can be assessed in several ways. First, we compared the number of polymorphic alleles at each locus between nocturnal and nonnocturnal asthmatics. At locus 16 in the nocturnal group with 23 patients there are 46 alleles, and as shown in Table III *A* 80.4% of these were Gly at this position, with the remainder being Arg. In contrast, the frequencies of these two alleles in the nonnocturnal cohort were nearly the same. The clustering of the Gly16 polymorphism with nocturnal asthma was highly significant by Fisher's Exact Test at P = 0.007 with an odds ratio of 3.8. The allelic frequencies of the two polymorphisms at position 27 were not different between the two groups (Table III *B*). The polymorphism at position 164 occurred only twice in the nonnocturnal group. Although there was a greater number of males in the nocturnal asthma group, this does not account for the observed increased allelic frequency of Gly16. For males and females in the nocturnal asthma group, the Gly16 allelic frequencies were 78.5 and 77.5%, respectively. In the nonnocturnal group, these frequencies for males and females were 55.5 and 54%. The presence of three Black subjects in the nonnocturnal group, as compared to none in the nocturnal group, did not favor the overrepresentation of the Gly16 allele in the nocturnal group. (Indeed, 5 of the 6 alleles at position 16 were Gly in the Black nonnocturnal asthmatics.).

Next, we compared the homozygous and heterozygous states of patients from each group. Regardless of how such analyses are performed, the results consistently reveal an overrepresentation of the Gly16 polymorphism in nocturnal asthma (Table IV). Initially we made the assumption that the homozygous (as compared to a heterozygous) genotype would have the greatest chance of segregating the two asthmatic phenotypes, and thus a valid comparison would be between those with or without nocturnal asthma and the homozygous polymorphisms, excluding those who are heterozygotes. For the polymorphisms at position 16 this comparison is shown in the upper portion (*A*) of Table IV. As can be seen, there was an overrepresentation of the homozygous Gly16 variant in the nocturnal asthma cohort. Of 18 patients with nocturnal asthma, 16 (88.9%) were homozygous for Gly16. On the other hand, there was about an equal distribution of homozygous Arg16 and homozygous Gly16 in the nonnocturnal group. This association of homozygous Gly16 with nocturnal asthma was significant at P = 0.046, with an odds ratio of 7.0. The inclusion of heterozygous patients in this comparison did not alter these results. The 3 × 2 contingency table for this comparison is shown in part *B* of Table IV. The Chi-squared Test for Trend indicated a significant (P = 0.017) linear trend among the categories. We also present a comparison based on the possibility that only the homozygous form of Gly16 imparts the nocturnal phenotype, with heterozygosity or homozygosity for Arg16 being insufficient. Such a comparison of homozygous Gly16 or "not homozygous Gly16" is shown in Table IV part *C*. The results again show an overrepresentation of Gly16 in the nocturnal asthmatic cohort. Of the 23 patients with nocturnal asthma 16 (69.6%) were

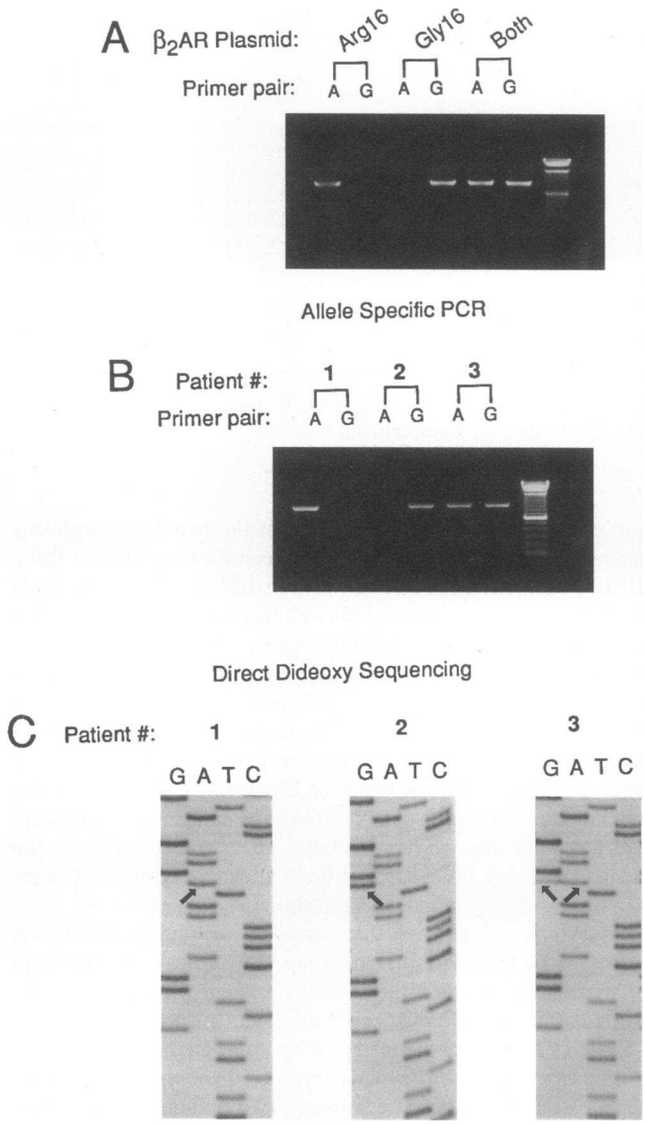

Figure 1. Identification of β_2AR polymorphisms using allele-specific PCR. *A* shows the specificity of the technique using plasmids with the indicated mutations. Primer pairs (labeled as *A* or *G*) were designed to provide PCR products when adenosine or guanine, respectively, are at nucleic acid position 46 of the antisense strand. *B* shows the technique applied to genomic DNA obtained from the blood of three patients who were homozygous for either Arg16 or Gly16, or heterozygous. Arrows indicate the polymorphisms as confirmed by dideoxy sequencing (*C*).

homozygous Gly16, while 8 of 22 (36.4%) of the nonnocturnal cohort were homozygous for Gly16. Thus the majority of patients with nocturnal asthma had the homozygous Gly16 polymorphism, and the majority of nonnocturnal asthmatics did not (i.e., had Arg at one or both alleles at position 16). These comparisons were significant at $P = 0.038$ with an odds ratio of 4.0. Further support that the Gly16 polymorphism is associated with nocturnal asthma is noted when patients are segregated based on a history of frequent nocturnal asthma rather than PEFR criteria. Of the 31 asthmatics with histories of nocturnal worsening, 23 (74.2%) had the homozygous Gly16 polymorphism, while among those without nocturnal histories only 1 of 14 (7.1%) was homozygous for Gly16 ($P < 0.0001$, odds ratio = 33).

Table III. Allele Frequencies of β_2AR Polymorphisms at Amino Acid Positions 16, 27, and 164 in Nocturnal and Nonnocturnal Asthma

	Nocturnal asthma No. of alleles	Nonnocturnal asthma No. of alleles	*P*-value	Odds ratio
	(percentage)	*(percentage)*		
(*A*)				
Gly16	37 (80.4)	23 (52.2)	0.007	3.8
Arg16	9 (19.6)	21 (47.8)		
(*B*)				
Glu27	23 (50.0)	23 (52.2)	> 0.05	NA
Gln27	23 (50.0)	21 (47.8)		
(*C*)				
Thr164	46 (100)	42 (95.4)	> 0.05	NA
Ile164	0 (0)	2 (4.5)		

Asthmatic phenotypes were determined by PEFR criteria as described in Methods.

Unlike what was observed at position 16, the numbers of homozygous Glu27, homozygous Gln27 and heterozygous Glu/Gln27 polymorphisms were no different between nocturnal and nonnocturnal asthmatics (Table IV *D*). The polymorphism at amino acid 164 (Ile164) was rare, with two nonnocturnal asthmatics found to have this polymorphism in the heterozygous state (Table 4 *E*). In some patients, these polymorphisms occurred together. As we have recently shown, though, the pres-

Table IV. Distribution of Homozygous and Heterozygous β_2AR Polymorphisms at Amino Acid Positions 16, 27, and 164

Genotype	Nocturnal asthma	Nonnocturnal asthma	*P*-value	Odds ratio
	(No. of patients)	*(No. of patients)*		
(*A*)				
Homozygous Gly16	16	8	0.046	7.0
Homozygous Arg16	2	7		
(*B*)				
Homozygous Gly16	16	8		7.0
Heterozygous Arg/Gly16	5	7	0.017	2.5
Homozygous Arg16	2	7		1.0
(*C*)				
Homozygous Gly16	16	8	0.038	4.0
Not homozygous Gly16	7	14		
(*D*)				
Homozygous Glu27	7	5		
Heterozygous Glu/Gln27	9	13	> 0.05	NA
Homozygous Gln27	7	4		
(*E*)				
Homozygous Thr164	23	20		
Heterozygous Thr/Ile164	0	2	> 0.05	NA
Homozygous Ile164	0	0		

Asthmatic phenotypes were determined by PEFR criteria as described in Methods. NA, not applicable.

ence of Gly at position 16 imparts enhanced downregulation regardless of whether the amino acid at position 27 is Gln or Glu (11), so comparisons of the position 16 polymorphisms independent from those at position 27 are appropriate. But, we would expect in a sufficiently large population that the combination of both Gly16 and Glu27 homozygous polymorphisms would be overrepresented in nocturnal asthma. We compared the number of patients who were homozygous for both Gly16 and Glu27 in the two groups and found the combination present in 7 (30.4%) nocturnal and 4 (18.2%) nonnocturnal asthmatics. Due to the small sample sizes, these apparent differences do not reach statistical significance, but do support the above notion. The Ile164 polymorphism (which was found in only two subjects in the heterozygous state) occurred together with the Gly16 polymorphism in both subjects.

To explore whether the Gly16 polymorphism was merely a reflection of more severe asthma, the percent predicted FEV_1 for those patients who did or did not have the homozygous Gly16 polymorphism (regardless of their nocturnal vs nonnocturnal phenotype) were compared. These values, although clearly trending towards a lower predicted FEV_1 percentage for the nocturnal group, were not statistically different, being 73.9±3.2% vs 82.5±3.5%, respectively, ($P > 0.05$). In addition, we assessed the prevalence of Gly16 in these asthmatics based on whether they are defined as mild ($FEV_1 > 70\%$ predicted [16]) or moderate, and did not find an overrepresentation of the polymorphism in the moderately severe group. Out of the 13 moderate asthmatics as so defined, 8 (61.5%) had the Gly16 polymorphism, and of the 32 mild asthmatics, 16 (50%) had the polymorphism ($P > 0.05$). Finally, of those originally assigned to the nonnocturnal cohort by the PEFR criteria, but who had the homozygous Gly16 polymorphism, 7 of 8 (87.5%) had positive histories of nocturnal asthma, whereas in this same group in those without homozygous Gly16 only 1 of 14 (7.1%) had positive nocturnal histories ($P = 0.0002$).

Discussion

Nocturnal worsening of airway obstruction in asthmatics is a well-documented phenomenon. Turner-Warwick in a large population study found that 39% of outpatient asthmatics had subjective nightly worsening of symptoms (17). The importance of this nighttime worsening stems from the fact that in addition to sleep disturbances, ~ 70% of deaths due to asthma occur during sleep-related hours (18). Also, ~ 80% of respiratory arrests due to asthma occur during the same period, with the single most important risk factor for such crises being diurnal variation in PEFR (19). The pathophysiologic mechanism of nocturnal asthma is not clear but such factors as hormonal circadian rhythms (5, 6, 8, 20), nocturnal worsening of inflammation (8, 21), and autonomic nervous system dysfunction (5–8) have been implicated in previous studies. Decreases in endogenous cortisol, which may result in increased inflammation and decreased β_2AR expression, have been the subject of several investigations (7, 8, 20, 22). A circadian decrease in plasma cortisol levels (with the nadir being around midnight) is observed in normal subjects (20). An exaggerated decrease in plasma cortisol has been reported in patients with nocturnal asthma (20, 22). Others, however, report no such differences between normal subjects, nocturnal asthmatics, and/or nonnocturnal asthmatics (7, 8). Differences in plasma histamine have also been reported (6), but again this is not a universal finding (8). An increased vagal activity, which promotes bronchoconstriction, has also been observed in nocturnal asthma (5). Diurnal variations in plasma catecholamine levels have been examined by a number of investigators with variable findings. Some have reported no differences in catecholamine levels or their diurnal variation between asthmatics with nocturnal worsening of PEFR and either nonnocturnal asthmatics or normal subjects (7, 20). Others have shown differences in epinephrine or norepinephrine levels between nocturnal and nonnocturnal asthmatics, and correlations between pulmonary function and fluctuations in plasma epinephrine (5, 6, 8). We have considered the possibility that a decrease in another component of this signal transduction cascade, namely the receptor itself, may have a role in the pathogenesis of nocturnal asthma.

We have previously examined nocturnal changes in β_2AR expression as a factor in nocturnal asthma (7). In those studies, mononuclear and polymorphonuclear leukocyte β_2AR were used as surrogates for β_2AR expressed on bronchial smooth muscle and other cell types relevant to asthma. β_2AR density and function were determined in normal subjects, nocturnal asthmatics, and nonnocturnal asthmatics at 4:00 p.m. and 4:00 a.m. In both cell types, β_2AR receptor density decreased at 4:00 a.m. by ~ 40% as compared to 4:00 p.m. in nocturnal asthmatics. No such nocturnal downregulation was observed in normal subjects or nonnocturnal asthmatics. Consistent with this downregulation, isoproterenol-stimulated cAMP production in cells from the nocturnal asthmatics was depressed at 4:00 a.m. compared to 4:00 p.m. (7). The mechanism responsible for this β_2AR downregulation found with nocturnal asthmatics, however, has remained unknown.

Our initial studies which lead to the identification of β_2AR polymorphisms were carried out in a group of normal and asthmatic subjects (9, 23). We found nine different polymorphisms within the coding block of the β_2AR at nucleic acids 46, 79, 100, 252, 491, 523, 1053, 1098, and 1239. Four of these (at nucleic acids 46, 79, 100, and 491) result in changes in the encoded amino acids at positions 16, 27, 34, and 164, respectively. In the β_2AR, amino acids 16 and 27 lie in the extracellular amino terminus, while amino acids 34 and 164 are in transmembrane spanning regions 1 and 4, respectively. We found an equal distribution of these polymorphisms between a group of normal controls and a heterogeneous group of asthmatics (9). Thus we concluded that genetic variation in the structure of the β_2AR was not a primary cause of asthma. Nevertheless, we did note that steroid-dependent asthmatics were more likely to have the Gly16 polymorphism. We found that polymorphisms at position 16 and 27 are relatively common (9). To maintain a common frame of reference, in our previous works we have defined the sequence of the human wild-type β_2AR as that which was reported with the original cloning of the human receptor (24) with Arg in position 16, Gln in position 27, and Thr in position 164. Thus the previous nomenclature that we have used: Arg16 → Gly, Gln27 → Glu, and Thr 164 → Ile (9–11, 23). However, even with the limited number of normal subjects that we have studied (9), it is clear that some of these polymorphisms are found frequently in the normal population and thus wild-type needs to be carefully defined. The most appropriate reference to these polymorphisms thus appears to be as we have used in the current work, i.e., Arg16, Gly16, etc.

To further explore the functional relevance of these polymorphisms, we mimicked these by site-directed mutagenesis of the wild-type β_2AR cDNA and expressed these in Chinese ham-

ster fibroblasts which normally do not express β_2AR. We found that the substitution of Ile for Thr at amino acid position 164 resulted in an ~ 3-fold decrease in agonist binding affinity and ~ 50% depression of agonist and nonagonist dependent coupling to adenylyl cyclase (10). In our recombinant expression system, substitution of Gly for Arg at position 16, Glu for Gln at position 27, or the combination of both, did not affect agonist or antagonist binding, sequestration, or coupling, but in contrast imparted altered agonist-promoted downregulation of the receptor (11). Glu at position 27 with Arg in position 16 resulted in a receptor that was virtually refractory to long-term (24 h) agonist-promoted downregulation of receptor number. On the other hand, the presence of Gly at position 16, regardless of whether a Glu or Gln is present at position 27, results in a more profound degree of long-term agonist-promoted downregulation as compared to Arg16 (11). In these latter studies, however, we did not assess the relative functional consequences of differences in these degrees of downregulation. The mechanisms of how these amino-terminal polymorphisms impart different agonist-promoted regulation are not fully known, but an altered degradation process of these receptor proteins appears to be the primary mechanism associated with these two genotypes (11). The rare (~ 1%) polymorphism at position 34 (substitution of Met for Val) confers no detectable alterations in receptor binding, coupling, or regulation (Green S. A., and S. B. Liggett, unpublished results).

Given our previous observation regarding the downregulation of β_2AR at 4:00 a.m. in nocturnal asthmatics (7), and the above findings of altered downregulation imparted by some β_2AR polymorphisms, we were prompted to examine the β_2AR genotype in two groups of asthmatics who did or did not have nocturnal asthma. We found a significant overrepresentation of the Gly16 polymorphism in the nocturnal asthmatic group. On the other hand, the frequency of the polymorphism at amino acid 27 was not different between the two groups. The polymorphism at position 164 was too infrequent to be considered (Table III). The Gly16 polymorphism is indeed the one that exhibits enhanced downregulation in recombinant studies (11). Our findings thus suggest that a contributing factor to the pathophysiology of nocturnal asthma is the presence of the Gly16 β_2AR polymorphism.

The assignment of asthmatics to the nocturnal vs nonnocturnal group was based on the overnight fall in PEFR, which was 34.0±2.0% in the nocturnal group and 2.3±0.8% in the nonnocturnal group. Daytime pulmonary function trended towards being worse in the nocturnal group as compared to the nonnocturnal group (Table II). Consistent with this is the observed greater use of theophylline and inhaled corticosteroids in the nocturnal group. Importantly, the use of β-agonists was the same in both groups. We do not believe however, that our findings should be interpreted simply as an association of the Gly16 genotype with more severe asthma. We base this on several factors. First, if one analyzes post hoc the mean fall in PEFR in patients based strictly on those who did or did not have the homozygous Gly16 polymorphism, there is a significant difference (24.0±3.4% vs 12.3±3.9%, $P = 0.017$) between these two groups. Furthermore, in those patients who were classified as nonnocturnal based on PEFR measurements but who had the Gly16 genotype, 7 of 8 were found to have strong histories of nocturnal symptoms in the past year. Conversely in those not homozygous for the Gly16 genotype only 1 of 14 had a history of nocturnal asthma. Lastly, separating all the subjects into mild vs moderate asthma, there was no significant differences between the two groups in the frequency of the Gly16 polymorphism.

β_2AR are localized in a number of regions of the lung which may be involved in the pathophysiology of asthma (reviewed in 25–27). Clearly the most well-characterized of these is bronchial smooth muscle, where activation of β_2AR results in relaxation and increased airway diameter. While such bronchodilatation is the primary therapeutic goal for administering β-agonists, the role of the β_2AR in the absence of exogenous agonist in modulating airway tone is not clear. Relevant to asthma, β_2AR are also present on epithelial cells, submucosal glands, and vascular smooth muscle. In addition, these receptors are expressed on a number of immune cells such as lymphocytes, neutrophils, and macrophages. From many studies in humans, it is clear that β_2AR expression and function is dynamically regulated by a number of factors (28). The stimulus for induction of downregulation of the Gly16 polymorphic form of the β_2AR in nocturnal asthmatics is not clear. Both nocturnal and nonnocturnal asthmatics have been reported to have peak plasma catecholamine levels in the late afternoon to early evening (6, 8). Given the typical time course required for β_2AR downregulation, such a peak in endogenous agonist may be sufficient to induce downregulation of the more sensitive Gly16 polymorphism in nocturnal asthmatics. Cortisol also regulates expression of β_2AR and undergoes a diurnal variation (20). We do not know, however, whether the Gly16 polymorphism is regulated differently by corticosteroids at this time.

It is important to note that the Gly16 polymorphism of the β_2AR appears from our study to be associated with one particular phenotype of asthma. It is clear from several studies that asthma and/or atopy may be the result of multiple gene defects. Atopy or elevated IgE levels have been reported to segregate with chromosome 11q13 (29), with a specific polymorphism of the IgE receptor (30), with a region near the IL-4 locus on chromosome 5q31.1 (31), and another region (the D5S436 marker) on chromosome 5 (32). It is intruiging to note that one of the few known genes near this latter marker is the β_2AR. Further studies saturating this region with additional markers will be necessary to determine the region of greatest segregation. Given the above results coupled with our current report, the involvement of β_2AR polymorphisms in a multigene defect scenario for asthma or certain asthmatic phenotypes appears reasonable.

In conclusion, we have found an association between the presence of nocturnal asthma and a polymorphism (Gly16) of the β_2AR. In recombinant studies, this polymorphism imparts enhanced downregulation of the receptor, which is consistent with the nocturnal downregulation of β_2AR and the depressed response to β-agonists observed in nocturnal asthma. While there are clearly other processes that also participate in nocturnal asthma pathophysiology, the correlation of the results of genetic, physiologic, and pharmacologic studies are consistent with the Arg16 β_2AR polymorphism being an important factor in this subset of asthmatics.

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