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Tachyphylaxis to β_2-agonists in Spanish asthmatic patients could be modulated by β_2-adrenoceptor gene polymorphisms

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Summary
Background: The study of determinants of asthma is a subject of much interest currently, especially the pharmacogenetic aspects of asthma management. Genetic polymorphisms affecting amino-acids at positions 16 and 27 within β_2-adrenoceptor (β_2AR) gene have been implicated in the asthma phenotypes and influence on the variability observed in response to use of bronchodilator agents used in the treatment of asthma. Whether these polymorphisms alter the bronchoprotection response to β_2-agonist treatment in Spanish asthmatic population is unknown. The aim of this study was to investigate whether genetic polymorphisms within β_2AR gene modulate the clinical outcomes of the individual response to β_2-agonist therapy and the development of desensitization in Spanish asthmatic patients.
Methods: In a prospective, case-control study were included 80 asthmatic patients. Based on the standard criteria, patients were classified into two groups: patients with tachyphylaxis and good responders to β_2-agonist therapy. DNA samples were genotyped for the $Arg^{16}Gly$ and $Glu^{27}Gln$ alleles within the β_2AR gene as well as in 64 control samples from blood donors.
Results: Arg^{16} allele was slightly more frequent within the group with tachyphylaxis ($P = 0.039$), whereas Gly^{16} allele carriers were overrepresented within the group of good responders (59.7%, $P = 0.028$). On the other hand, the allele frequency of Gln^{27}

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and the proportion of Gln^{27} carriers was higher within the group with tachyphylaxis ($P = 0.010$ and 0.049, respectively) and Glu^{27} allele carriers were overrepresented within the group of good responders ($P = 0.026$). The Arg^{16} and Gln^{27} alleles were in strong linkage disequilibrium across this locus, resulting in the occurrence of disease haplotype.

Conclusions: The predisposition to develop tachyphylaxis in our population seems to be linked to the Arg^{16} and Gln^{27} alleles and to the Arg^{16}/Gln^{27} risk haplotype (positive association between the presence of the Arg^{16} and Gln^{27} alleles and tachyphylaxis). The Arg^{16} allele is perhaps overrepresented due to the strong linkage disequilibrium between both polymorphisms. The presence of the Glu^{27} allele seems to be a protective factor against tachyphylaxis in this cohort study.

Introduction

Beta$_2$-adrenergic receptor (β_2AR) is a cell surface protein expressed on many lung cell types relevant to asthma, i.e. airway epithelial cells, bronchial smooth muscle, pre-synaptic nerve terminals and eosinophils. β_2AR is coupled to a G-protein that activates adenylate-cyclase resulting in the formation of AMPc which, as a second messenger, activates protein-kinases that mediate different responses on different cell types.

Beta$_2$-adrenoceptor agonists are used by virtually all patients as rescue bronchodilator agents and recommended as first-line therapy for the relief of bronchoconstriction in patients with asthma due to its protective effects against direct and indirect bronchoconstrictor stimuli shown by short- and long-acting β_2-agonists. Regular treatment with both short- and long-acting β_2-agonists may result in β_2AR desensitization, a phenomenon termed "*tachyphylaxis*" which is associated to the loss of functional antagonism to bronchoconstrictor stimuli, although the relevance of this phenomenon in terms of long-term asthma control remains unclear.

There has been a debate on whether a subgroup of asthmatic patients does not benefit from regular β_2-agonist therapy. One potential source of the individual variability to β_2-agonist response could be DNA sequence variations (polymorphisms) within β_2AR gene. Within the coding region of the human β_2-adrenoceptor gene itself, until now, nine SNPs have been identified,[1] five of which are degenerate. Non-degenerate polymorphisms result in single amino acid substitutions in codon 16 (Arg^{16}–Gly), 27 (Gln^{27}–Glu), 34 (Val^{34}–Met), and 164 (Thr^{164}-Ile). Amino-acids 16 and 27 lie in the extracellular domain and amino-acids 34 and 164 in the transmembrane spanning regions. The relative frequencies of these polymorphisms are similar in normal and asthmatic population and this does not seem to determine the development of the asthmatic phenotype, although it may contribute to its modification.[2]

Several recent studies have related both Arg^{16}–Gly and Gln^{27}–Glu polymorphisms to different clinical and analytical data. Taking into account, as expected, that there is a strong linkage disequilibrium between these polymorphisms, it is difficult to assign an effect to a single allele in association studies. Gly^{16} has been associated with nocturnal asthma,[3] lower response to inhaled β_2-agonists in children and increased bronchial hyperresponsiveness (BHR) to histamine. At codon 27, the Gln polymorphism is associated with elevated IgE levels while the Glu^{27} allele is associated with lower airways hyperreactivity to methacoline in asthmatics families.[4] In a recent study, Joos et al.[5] found a negative association (protective effect) between heterozygosity at position 27 and an accelerated rate of decline in lung function in smokers.

Homozygous individuals for Arg^{16} showed an increased response to albuterol both in asthmatic and non-asthmatic children and the effect was independent of baseline lung function and ethnic origin.[6] Furthermore, "in vitro" studies showed that Gly^{16}Arg and Glu^{27}Gln polymorphisms markedly altered the agonist promoted receptor down-regulation and functional desensitization of β_2AR.[7]

The phenotypic consequences of a given single polymorphism could be at the same time modified by interactions with other polymorphisms in the promoter or coding region.[8] So, the variability of the response to inhaled β_2-agonists could be better predicted by β_2AR extended haplotypes.

Our hypothesis was that the polymorphisms within β_2AR gene could modulate the bronchodilator response to inhaled β_2-agonists by inducing tachyphylaxis in the Spanish asthmatic population.

The aim of this study is to compare the relative frequency of the two main β_2AR polymorphisms between asthmatic patients and control subjects

and to assess the relationship between different combinations of these alleles and β_2AR haplotypes with the clinical outcomes of the response to inhaled β_2-agonists use and the development of tachyphylaxis.

Methods

Subjects

The patients were enrolled into a prospective, case-control, randomized, study design. Eighty patients (39 males and 41 females) who had an established diagnosis of stable mild-to-moderate persistent asthma. All patients were participants in the Allergy Unit (University Hospital Pío del Rio Hortega, Valladolid, Spain) (mean age: 29.9 year; range 14–64 years). All cases showed serum IgE levels above 100 IU/ml and a positive skin prick test for at least one pneumoallergen.

All patients included in this study had been diagnosed as having atopic asthma since at least past six months. Patients were considered to be atopic if they had at least one skin test measuring 3 mm or more in diameter.

Data confirming obstruction included increased residual volume and airway resistance, uneven distribution of ventilation and decrease of FEV_1 according to American Thoracic Society (ATS) criteria.[9] Reversibility of airway obstruction in response to administration of bronchodilator aerosol (salbutamol 400 μg) was measured following standard method from ATS.[9,10] A positive reversibility test was defined by an increase of FEV_1 higher than 15% after inhalation of 400 μg of salbutamol and measured as:

$$\%\,\text{reversibility} = \frac{\text{highest FEV}_1\text{ after salbutamol} - \text{highest FEV}_1\text{ before salbutamol}}{\text{highest FEV}_1\text{ before salbutamol}} \times 100.$$

Tachyphylaxis was defined as an absence of response to inhalation of short-acting β_2-agonists during a period of three months and measured in at least four occasions, and a lack of β_2-agonists mediated bronchoprotection on bronchial responsiveness induced by bronchial challenge with specific allergen performed by using the Cockcroft technique.[11] Based on these criteria, patients were classified in two groups:

Group with tachyphylaxis: Twenty-three out of 80 (28.8%) (10 males and 13 females) (mean age:31.2 year; range 14–59 years) with tachyphylaxis to inhaled β_2-agonists. None patients received inhaled steroid therapy in order to avoid confounded results in the reversibility to bronchodilator.

Group of good responders: Fifty-seven patients (29 males and 28 females) (mean age: 29.4 year; range 15–64 years) with good response to inhaled β_2-agonists use. Steroid therapy was not needed in this group of patients.

β_2-agonists are used on demand and the dosage is adjusted by the patients themselves, with a limit of 6 puffs per day. At the end of the study, the group of patients with tachyphylaxis were taking a higher dose of β_2-agonists. The mean number of daily puffs of the bronchodilator in the "good responder" group was 2 puffs/bid, while patients suffering from tachyphylaxis this was 6 puffs/bid.

Patients were excluded of study if they had been treated with oral corticosteroids in the past 6 months or had a history of recent respiratory tract infection within the past 3 months, or had smoked cigarettes within the previous 12 months.

Sixty-four samples from blood donors were studied to assess the frequency of the β_2AR alleles in our population. Asthma patients cannot be blood donors in our Hospital. Individuals from both groups (asthma patients and controls) were white Caucasian Spanish and did not include individuals from different ethnic background. All of the patients gave written informed consent for the study, which was approved by the Valladolid University Hospital Committee for Medical Research Ethics.

Genotyping methods

Genomic DNA was extracted from whole peripheral blood samples using standard techniques. Genotyping of the polymorphisms at positions 16 and 27 within β_2AR gene was performed by a combination of primer-induced restriction site assay for $Arg^{16}Gly$ polymorphism and restriction fragment assay for $Gln^{27}Glu$ polymorphism as previously described elsewhere.[6]

The β_2AR-27 alleles were typed using enzyme *Bse*XI (Fermentas®), an isoschizomer of *Bbv* I. In this case the PCR amplimers were digested at 65 °C for 3 h.

The products of each PCR reaction were resolved by electrophoresis on 3% agarose gels stained with ethidium bromide. Template-free and known genotype controls were included in each experiment.

Genotypes were scored without knowledge of the phenotypes by two independent observers. The samples were re-genotyped if there was any disagreement concerning the genotype.

Statistical analysis

The results are presented as haplotype and allele frequencies for both asthmatic (tachyphylaxis and good responders) groups. The differences in haplotypes and allele frequency between the two groups were assessed by Pearson's χ^2 tests for 2×2 contingency tables as previously described.[12] All tests were performed using the JMP Statistics software package (SAS Institute Inc). A P value of <0.05 was considered to be statistically significant. All values are expressed as mean (SE).

Haplotypes frequencies were estimated using the expectation maximisation (EM) algorithm since haplotypes could not be discerned directly from double heterozygotes. The Arlequin software package was used to perform these estimations, to test for linkage disequilibrium, and for Hardy-Weinberg equilibrium. The analysis of the haplotypes defined by the two β_2AR polymorphisms was performed by using the Haplo-Score Package.[13]

Results

The allele frequencies found in our Spanish population were 0.51 for Gly^{16} and 0.63 for Gln^{27}, thus the results were similar to those previously reported by other authors[25] for Caucasian populations.

The study of homozygous samples for at least one polymorphism showed a strong linkage disequilibrium ($D' = 100\%$, $P < 0.000001$) given that 75.0% of Arg^{16} chromosomes carried the Gln^{27} allele; and this linkage was also found both in asthmatic patients (73.9%) and controls (76.1%) without significant statistical differences.

Single-locus analysis

Allele frequencies (Table 1) found were almost similar in asthmatic patients and controls for both Arg^{16} (43.1% and 49.3%, $\chi^2 = 1.064$; $P = 0.302$; respectively) and Gln^{27} (58.1% and 62.5%, $\chi^2 = 0.739$; $P = 0.390$; respectively).

Genotypic frequencies of $Arg^{16}Gly$ and $Gln^{27}Glu$ polymorphisms were in Hardy-Weinberg equilibrium and did not show statistically significance differences between both groups.

Individuals carrying the Gly^{16} allele were more frequent within the group of good responders than in the group with tachyphylaxis (59.7% vs. 39.1%, respectively; $\chi^2 = 6.482$; $P = 0.028$) and this is caused by the increased number of heterozygous $Arg^{16}Gly$ ($n = 34$). In fact, 51/57 (89.5%) patients without tachyphylaxis carrying the Gly^{16} allele (Table 1).

Otherwise, the Gln^{27} allele was overrepresented within group of patients with tachyphylaxis when allele frequencies were compared with good responders (73.9% vs. 51.8%, respectively; $\chi^2 = 9.509$,

Table 1 Distribution of the genotypes identified among the groups studied.

	CODON 16				CODON 27			
	FMA*	Genotypes			FMA*	Genotypes		
Population#	Arg^{16}	Arg/Arg	Arg/Gly	Gly/Gly	Glu^{27}	Gln/Gln	Gln/Glu	Glu/Glu
Asthmatic patients	69/160	13	43	24	67/160	27	39	14
($n = 80$)	(43.1%)	(16.2%)	(53.7%)	(30.1%)	(41.9%)	(33.8%)	(48.7%)	(17.5%)
Tachyphylaxis	23/46	7	9	7	12/46	12	10	1
($n = 23$)	(50.0%)	(30.4%)	(39.1%)	(30.5%)	(26.1%)	(52.2%)	(43.5%)	(4.3%)
Good responders	46/114	6	34	17	55/114	15	29	13
($n = 57$)	(40.4%)	(10.5%)	(59.7%)	(29.8%)	(48.2%)	(26.3%)	(50.9%)	(22.8%)
Controls	63/128	17	29	18	48/128	30	20	14
($n = 64$)	(49.3%)	(26.6%)	(45.3%)	(28.1%)	(37.5%)	(46.9%)	(31.2%)	(21.9%)
		$\chi^2 = 1.064$, $P = 0.302^+$				$\chi^2 = 0.739$, $P = 0.390^+$		

Definition of abbreviations: Arg = Arginine; Gly = Glycine; Gln = Glutamine; Glu = Glutamic acid.
Number of subjects with polymorphisms at positions 16 and 27 of β_2AR gene.
*FMA: frequency of the minor allele.
#Values are shown as number of subjects (%), unless otherwise indicated.
+Hardy-Weinberg equilibrium test values.

$P = 0.010$). Gln^{27} allele carriers were more frequent within group of tachyphylaxis (95.7%) while Glu^{27} allele carriers were more frequent within group of good responders (73.7%). Fourteen asthmatic patients were Glu^{27}/Glu^{27} homozygous (17.5%), only one of them was identified within the group with tachyphylaxis (4.3%) (Fig. 1).

Haplotype analysis

The analysis of the distribution of β_2AR haplotypes within the stratified population showed a global statistical ($P = 0.078$). The Arg^{16}/Gln^{27} haplotype was more frequent within the group of patients with tachyphylaxis (26.1%, $P = 0.025$). Six out of 23 (26.1%) patients with tachyphylaxis and 1/57 (1.8%) good responders were homozygous for this haplotype (Table 2). Between good responders, the most frequent haplotype was Gly^{16}/Glu^{27}, but was not significant statistical (33.3%, $P = 0.087$). In Fig. 2, the frequencies (%) of the four haplotypes both in good responders vs. patients with tachyphylaxis are shown. Nineteen good responders and 4 patients with tachyphylaxis were double heterozygous and the linkage phase of both polymorphisms could not be settled (Table 2).

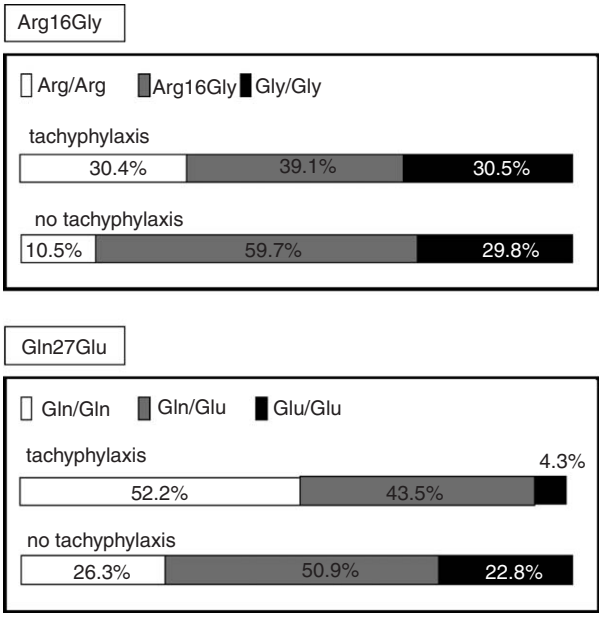

Figure 1 Gly^{16} and Glu^{27} alleles carriers were over-represented within the group of patients with no tachyphylaxis to β_2-agonists ($P = 0.028$ and 0.026, respectively).

Discussion

β_2AR gene maps within the chromosomal region 5q31–33.[14] This region contains many candidate genes which may be involved in the pathogenesis of asthma and atopy. Several studies have failed to demonstrate any relationship between β_2AR alleles and predisposition to asthma.[15]

The results of the present study showed that the allele frequencies were almost similar in asthmatic patients and in controls. The distribution of the genotypes were in Hardy-Weinberg equilibrium and not significant differences were found between both groups. These data agree with previous reports[7,15] and confirm the absence of association between β_2AR alleles and susceptibility to asthma.

The dosage of β_2-agonists varies depending on demand by each patient. As explained above, at the end of the study, patients with tachyphylaxis

Table 2 Observed frequencies of the β_2AR gene haplotypes for polymorphisms at positions 16 and 27 among asthmatic patients with tachyphylaxis and good responders to β_2-agonist therapy.

β_2AR Gene haplotypes	Good responders ($n = 57$)		Tachyphylaxis ($n = 23$)	
	n	(%)	*n*	(%)
1–1	1	1.8	6	26.1
1–2	4	7.0	1	4.3
1–3	10	17.5	4	17.4
1–4 +2–3*	19	33.3	4	17.4
2–2	1	1.8	0	0
2–4	5	8.8	1	4.3
3–3	4	7.0	1	4.3
3–4	6	10.5	6	26.1
4–4	7	12.3	0	0

Nomenclature of the β_2AR gene haplotypes determined (used): Allele 1: Arg^{16}/Gln^{27}, Allele 2: Arg^{16}/Glu^{27} , Allele 3: Gly^{16}/Gln^{27}, Allele 4: Gly^{16}/Glu^{27}.

*The linkage disequilibrium phase is unknown in double heterozygous patients.

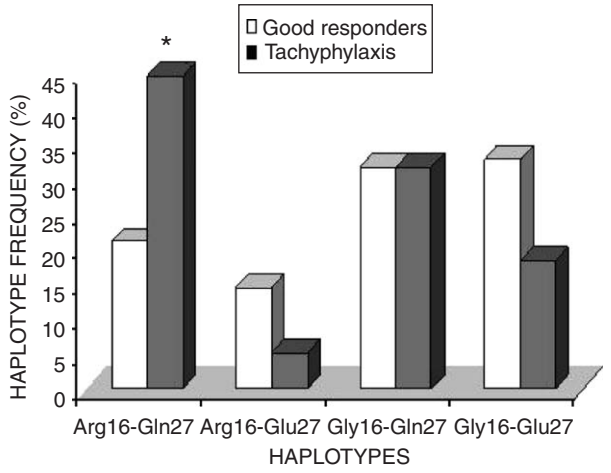

Figure 2 Haplotype frequencies in β_2AR genes. Linkage phase of the studied polymorphisms could not be settled in 38 chromosomes from patients with good response and in 8 chromosomes from patients with tachyphylaxis. $^*P = 0.025$ Student's test.

received higher doses than good responders. Although it could be suggested that the tachyphylaxis to β_2-agonists may be due to the dosage taken by the patients, we think that this is actually the consequence of the progressive loss of sensitivity to β_2-agonists.

It is noteworthy to mention the clear difference found between asthmatic patients and good response and those with tachyphylaxis to β_2-agonists. The risk of tachyphylaxis was higher in Gln^{27} allele carrying patients. Forty-four out of 57 patients (77.2%) within the group of good responders and 22/23 (95.7%) individuals within the group with tachyphylaxis carried at least one Gln^{27} allele ($P = 0.049$). This risk seems to be higher in homozygous (Gln^{27}/Gln^{27}) since 12/23 (52.2%) patients with tachyphylaxis and 15/57 (26.3%) good responders were Gln^{27} homozygous (52.2% vs. 26.3%, $P = 0.027$).

Our results suggest that the predisposition to desensitization in our population could be associated to both Arg^{16} and Gln^{27} variants, although the later polymorphism (Gln^{27}) seems to play a more important role, bearing in mind that it is difficult to assign a causative role to any single allele due to strong linkage disequilibrium exists between these promoter polymorphisms (and those within the coding region or the immediate region of the β_2AR gene), resulting in the occurrence of several common haplotypes.

In fact, in our study, the risk of developing tachyphylaxis seems to be linked to haplotype Arg^{16}/Gln^{27}, in agreement to Drysdale et al.[8] which performed a comprehensive survey of β_2AR haplotypes and showed that the Arg^{16}/Gly^{16} and Gln^{27}/Glu^{27} polymorphisms are sufficient to distinguish the three common haplotypes in white subjects. Another seven haplotypes exist in the white population but they are all rare and the combined frequency of these haplotypes is only 5.5%. It is therefore unlikely that additional haplotype data would substantially change our results, although the global statistic is only indicative of a weak association. This result suggests than it would be desirable to develop simple but robust molecular methods to determine the haplotype structure of Spanish asthmatic patients.

Our study is discordant with a previous study that reported linkage between predisposition to bronchodilator desensitization and alleles Gly^{16} and Glu^{27}.[16] The different findings could be explained since this study is quantitative measured as (%) of desensitization and carried out in 22 asthma patients. Moreover, most patients were receiving steroid therapy which could be a modifier of the response to β_2-agonists.

According to these results, it is clear that variants within the β_2AR gene could modify the response to β_2-agonist therapy in our Spanish population.

Although it is difficult to explain the different behaviour of the variants at codons 16 and 27, our data corroborates a previous study of the functional effects in lung mast cells where Gly^{16} and Glu^{27} isoforms within β_2AR gene were resistant to agonist-induced desensitization, as expressed by isoproterenol-induced inhibition of histamine release. Here the polymorphism at position 27 seems to play the most important role.[17]

The role of β_2-adrenoceptor regulation in asthma is unclear yet. While tachyphylaxis to the bronchodilator effects of β_2-agonists occurs in non-asthmatic individuals, it has been difficult to demonstrate in asthmatics.[18–22] However, tolerance to the broncho-protective effects of β_2-agonists in asthma has been demonstrated.[23–25] Thus, the mechanisms of β_2-adrenoceptor regulation in asthma appear to differ between cell types and may be altered by disease states.[26] It has been reported that asthmatic subjects homozygous for Gly^{16} demonstrate greater tachyphylaxis to the long-acting β_2-agonist formoterol.[16]

In summary, this is the first report to address the possible effect of these β_2AR gene polymorphisms on the response to regular use of β_2-agonists in the Spanish asthmatic population. Nevertheless, results of the associations shown spite of the relatively small size of the sample, are significant and they can be therefore relevant for respiratory clinicians and geneticists in order to make out whether a patient will be responsive to β_2-agonist treatment, or

may experience adverse events in association with β_2-agonist therapy, as well as to the current asthma management guidelines, since β_2-agonists represents the first-line therapy in treatment of asthma.

Further studies involving larger patient numbers are needed to assess the association between asthma phenotypes and haplotypes analysis studied to date, the role played by β_2AR isoforms in the development of tachyphylaxis to β_2-agonists, as well as to define the most accurate therapy for the individual asthma patient in our Spanish population. Up to date, it is also truth that many questions related to the risk of desensitization remain unclear.

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