

Original article

The effect of β 2-adrenoceptor haplotypes on bronchial hyper-responsiveness in patients with asthma

Background: The β 2-adrenoceptor exhibits genetic polymorphism which may be clinically relevant in terms of treatment response or bronchial hyper-responsiveness (BHR). The combined effect of these genotypes, or the haplotype, has not been fully characterized in terms of BHR.

Methods: We performed a retrospective analysis of the effects of haplotypes of amino acid substitution at position 16 (Gly/Arg) and position 27 (Gln/Glu) on spirometry and BHR to methacholine and adenosine monophosphate (AMP) in 594 asthmatic patients.

Results: There was a significant ($P < 0.05$) overall effect for forced expiratory volume (FEV_1) but not after correction for steroid dose and atopic status. There were no significant differences for BHR to methacholine or AMP between the different haplotypes and no difference between the numbers of patients with or without clinically relevant BHR. Methacholine PD20 geometric mean-fold difference was 1.63 (95% CI: 0.95–2.80) between Arg–Arg/Gln–Gln and Gly–Gly/Gln–Gln and 1.26 (95% CI: 0.75–2.11) between Gly–Gly/Gln–Gln and Gly–Gly/Glu–Glu.

Conclusions: The degree of BHR to indirect and direct stimuli does not differ between β 2-adrenoceptor haplotypes, and haplotypes cannot be used to predict BHR in patients presenting with asthma. Although β 2-adrenoceptor haplotypes do not predict BHR they may be important in predicting response to bronchodilator therapy.

A. M. Wilson¹, R. D. Gray², I. P. Hall³, B. J. Lipworth⁴

¹Biomedicine Group, School of Medicine, Health Policy and Practice, University of East Anglia, Norwich; ²Medical Genetics, Molecular Medicine Centre, Western General Hospital, Edinburgh; ³Department of Medicine, Division of Therapeutics, University Hospital, Nottingham; ⁴Asthma and Allergy Research Group, Department of Medicine and Therapeutics, Ninewells Hospital and Medical School, University of Dundee, Dundee, UK

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B.J. Lipworth
Professor of Allergy and Respiratory Medicine
Asthma and Allergy Research Group
Department of Medicine and Therapeutics
Ninewells Hospital and Medical School
University of Dundee
Dundee DD1 9SY
UK

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Background

The tendency to develop asthma is related to both environmental factors and genetic predisposition. Environmental factors include exposure to childhood infections and allergen, cigarette smoking and occupational exposure. Genetic factors, evidenced by greater incidence rates in family members of asthmatics, include polymorphisms of a number of genes involved in atopy, airway inflammation and bronchomotor tone including the β 2-adrenoceptor.

Polymorphisms of the β 2-adrenoceptor may be clinically relevant as β 2-receptor agonists are the mainstay of bronchodilator pharmacotherapy in the management of asthma. Several β 2-adrenoceptor polymorphisms occur at a high frequency, which would be relevant to a population as a whole, and have physiological effects *in vitro* and *in vivo* (1). The two most common polymorphisms in our population occur at position 16 and 27. Substitution of glycine (Gly) for arginine (Arg) at the 16th amino acid on the β 2-adrenoceptor results in greater susceptibility to receptor downregulation on Chinese hamster fibroblasts

and human smooth muscle cells following prolonged exposure to isoproterenol. The substitution of glutamic acid (Glu) for glutamine (Gln) at the 27th amino acid has the opposite protective effect (2, 3). These translate into altered effects in response to therapy, which may be clinically relevant (4).

However, individual polymorphisms should not be considered alone. The combinations of multiple individual polymorphisms are referred to as the haplotype. It is, therefore, the haplotype rather than individual polymorphisms which is most relevant to the patient. For example, although the Gly16Glu27 haplotype results in less downregulation in human airway smooth muscle cells than Gly16Gln27 (5), the polymorphism at the position 16 is dominant over that at position 27 (6).

Bronchial hyper-responsiveness (BHR) is a characteristic feature of asthma. It is related to the degree of underlying mucosal inflammation (7) and correlates well with disease severity and requirement for therapy (8, 9), and is a key feature of the asthma phenotype. Previous data have shown a weak association of BHR with polymorphisms at 16 or 27 (10, 11), but there are few reports on the

effects of haplotypes. Therefore, we evaluated the effect of the $\beta 2$ -adrenoceptor haplotype on BHR in a sample of patients who were diagnosed with asthma.

Methods

Patients

Demographic details are illustrated in Table 1. All patients presented for screening with a clinical diagnosis of asthma, made in either primary or secondary care, and were taking at least one anti-asthmatic drug. They were recruited to the Tayside Asthma and Allergy Research Group for more detailed assessment and characterization prior to potential participation in subsequent clinical research studies. This assessment includes details of medication, spirometry, skin prick testing, challenge testing with methacholine and adenosine monophosphate (AMP) and genotyping for $\beta 2$ -adrenoceptor polymorphisms. The Tayside Committee on Medical Research Ethics provided ethical approval for this screening process, including $\beta 2$ -adrenoceptor genotyping, and all patients gave their written informed consent.

Bronchial challenge testing

All bronchial challenge tests were performed after subjects had withheld their short-acting bronchodilators for 6 h and long-acting bronchodilators for 48 h. Methacholine and AMP bronchial challenge tests were performed as previously described (12, 13). In brief, methacholine was administered in cumulative doses from 3.125 to 1600 μ g and AMP was administered in doubling concentrations from 0.09 to 800 mg/ml. The provocation dose/concentration required to cause a 20% fall in forced expiratory volume (FEV_1) was calculated by interpolation of the steep part of the log dose-response curve. If the FEV_1 did not fall by 20% after the maximum dose/concentration was administered, a value of 3200 μ g and 1600 mg/ml was assigned for methacholine and AMP respectively. In our laboratory, the cut-off value for methacholine is 500 μ g and for AMP is 200 mg/ml.

Identification of the $\beta 2$ -adrenoceptor polymorphisms and haplotype

$\beta 2$ -Adrenoceptor polymorphisms were identified as previously described (11). In brief, genomic DNA was extracted from whole

blood, and a 234 base-pair fragment generated by polymerase chain reaction (PCR) that spanned the regions of interest. The primers used were 5'-CCCAGCCAGTGCCTTACCT-3' and 5'-CCGTCTGCAGACGCTCGAAC-3'. The genotype was identified by allele-specific oligonucleotide hybridization using 19-mer probes homologous for the Arg-16, Gly-16, Gln-27, or Glu-27 forms of the receptor. A random selection of PCR fragments was also directly sequenced to confirm the specificity of genotype identification by allele-specific digonucleotide. Subjects were classified as having one of nine possible haplotypes from the combination of amino acids at location 16 and 27.

Spirometry

Spirometry was performed according to American Thoracic Society criteria (14) using a Vitalograph compact spirometer (Vitalograph Ltd, Buckinghamshire, UK) with a pneumotachograph head and pressure transducer which was calibrated daily using a precision syringe (Vitalograph Ltd).

Skin prick testing

Patients withheld anti-histamine medication for 4 days prior to skin prick testing. This was performed following a standard protocol (Allerga Pharma Testing Solution; Diagnostics Ltd, Notts, UK) using extracts to grass, trees and weeds, house dust mite, *Aspergillus*, feathers, cat and dog dander in addition to a positive (histamine) and negative control. Results were read after 10 min, a positive reaction being defined as a weal diameter of 2 mm greater than the negative control. For a given patient, the number of skin prick-positive reactions to allergen was taken as the atopic status.

Statistical analysis

Data for methacholine PD20 and AMP PC 20 were log transformed to normalize their distribution prior to analysis. Analysis of variance (ANOVA) was used to determine whether there was a significant difference between haplotypes for spirometry, methacholine bronchial challenge or AMP challenge. Analysis was performed for the whole group and for subgroups of patients with clinically relevant BHR to methacholine (< 500 μ g) or AMP (< 200 μ g), with steroid dose and atopic status used as co-factors. Analysis was also performed in the subgroup of patients (methacholine $n = 140$, AMP $n = 124$) who did not receive inhaled corticosteroid therapy. Chi-square test was used to determine whether there was a difference in the number of patients with each haplotype with BHR to

Table 1. Demographic details for the different $\beta 2$ -adrenoceptor haplotypes with details of number of patients (%), age (SEM), age range, number male, number taking inhaled corticosteroids (number on steroids), median steroid dose (range); mean (SEM) forced expiratory volume in 1 second (FEV_1), FEV_1 % predicted, mid-forced expiratory flow (FEF), FEF % predicted, and number of patients with more than 1 skin prick positive (total number of patients skin prick tested)

Haplotype	Number (%)	Age (years)	Age range	Male	Number on steroids	Inhaled steroids (μ g/day)	FEV_1 (l)	FEV_1 % predicted (%)	FEF (l/s)	FEF % predicted (%)	Skin >1 (total tested)
Arg-Arg/Gln-Gln	77 (13.0)	41.0 (1.6)	20-72	38	42	400 (100-1600)	3.1 (0.1)	90.03 (0.02)	2.8 (0.1)	69.2 (0.03)	39 (64)
Arg-Arg/Gln-Glu	8 (1.3)	36.1 (4.0)	23-50	4	7	600 (200-1600)	3.1 (0.3)	88.25 (0.06)	2.8 (0.3)	64.0 (0.05)	6 (7)
Arg-Gly/Gln-Gln	89 (15.0)	39.7 (1.5)	19-68	37	61	500 (100-3200)	2.9 (0.1)	87.52 (0.02)	2.8 (0.1)	69.0 (0.03)	60 (74)
Arg-Gly/Gln-Glu	191 (32.2)	43.0 (0.9)	19-72	85	111	400 (7-2000)	2.9 (0.1)	90.28 (0.01)	2.7 (0.1)	68.1 (0.02)	98 (133)
Arg-Gly/Glu-Glu	4 (0.7)	35.8 (6.5)	23-54	0	2	700 (400-1000)	2.8 (0.3)	95.00 (0.04)	2.7 (0.6)	71.8 (0.13)	3 (3)
Gly-Gly/Gln-Gln	28 (4.7)	44.3 (3.0)	23-68	11	14	400 (125-2000)	2.8 (0.1)	88.50 (0.04)	2.4 (0.2)	63.8 (0.05)	17 (22)
Gly-Gly/Gln-Glu	90 (15.2)	44.9 (1.6)	19-74	34	59	500 (100-2000)	2.6 (0.1)	85.07 (0.02)	2.3 (0.1)	61.2 (0.02)	54 (73)
Gly-Gly/Glu-Glu	107 (18.0)	41.1 (1.2)	21-69	48	63	400 (3-2000)	3.0 (0.1)	89.74 (0.02)	2.7 (0.1)	66.6 (0.03)	65 (86)
Total	594 (100)	42.1 (0.6)	19-74	257	359	400 (3-3200)	2.9 (0.0)	88.86 (0.01)	2.6 (0.0)	86.3 (0.01)	342 (462)

methacholine or AMP compared with those not demonstrating BHR. Multiple linear regression analysis was performed using the backward elimination method to assess predictors for methacholine PD20 and AMP PC20. PD20 and AMP PC20 were used as the dependent variables and FEV₁% predicted, atopy, haplotype and steroid dose were used as independent variables in the analysis. Analyses were performed using SPSS for Windows (Statistical Products and Service Solutions Inc., Chicago, IL, USA).

Results

Of the 1337 patients in the database, the polymorphisms of the β -adrenoceptor at codons 16 and 27 were known in 594 patients, permitting identification of the haplotype of two commonest forms of the β 2-receptor polymorphism. Of these 594 patients, 580 patients had measurement of FEV₁, 441 had a methacholine challenge test, 384 had an AMP bronchial challenge test and 333 had both challenges. There was an unequal distribution of haplotypes with no patient exhibiting the Arg-Arg/Glu-Glu haplotype (Table 1). There was a significant overall difference between haplotypes and FEV₁ ($P = 0.02$) but not FEV₁% predicted, FEF25-75, FEF25-75% predicted or steroid dose (Table 1). The Gly-Gly/Gln-Glu haplotype had a significantly ($P < 0.05$) lower FEV₁ than the Arg-Arg/Gln-Gln haplotype [95% confidence interval (CI) for difference 0.06 to 0.91 l], and Arg-Arg/Gln-Glu haplotype (95% CI for difference 0.003 to 0.79 l). However, this effect was not significant when steroid dose and atopic status were used as cofactors.

There was no significant difference for methacholine PC20 or AMP PD20 between the different haplotypes for all patients who had had these challenge tests. Likewise, there was no significant effect of haplotype in the subgroup of subjects who had a PD20 methacholine ≤ 500 μ g (266 patients) or PC20 AMP ≤ 200 mg/ml (169 patients) (Table 2). There was also no significant difference for methacholine PC20 ($P = 0.65$) or AMP PD20 ($P = 0.75$) between the different haplotypes for those patients not receiving inhaled corticosteroid therapy.

There was no significant difference between the number of subjects with a methacholine PD20 ≤ 500 μ g compared with those with PD20 > 500 μ g; or the number of subjects with an AMP PC20 ≤ 200 compared with those with a PC20 > 200 μ g for each haplotype. The geometric mean-fold differences for methacholine PD20 for the homozygous haplotypes in subjects with a methacholine PC20 ≤ 500 μ g and an AMP PD20 ≤ 200 mg/ml are shown in Table 3.

Haplotype was not shown to be an independent predictor of either methacholine or AMP challenge testing as this was eliminated from the multiple linear regression model. The only independent predictor for methacholine PD20 was percentage predicted FEV₁ with a regression equation of $\log \text{PD20} = 1.165 + 0.010 \times \text{percent predicted FEV}_1$. The only independent predictors for AMP PC20 were

Table 2. Number of patients (n), mean and standard error of mean (SEM) for all patients having methacholine and adenosine monophosphate (AMP) bronchial challenge and those patients with a methacholine PD20 ≤ 500 mcg and AMP PC20 ≤ 200 mg/ml for each β -adrenoceptor haplotype

Haplotype	All		≤ 500	
	n	Mean (SEM)	n	Mean (SEM)
Methacholine				
Arg-Arg/Gln-Gln	57	244.8 (10.8)	32	37.3 (2.0)
Arg-Arg/Gln-Glu	6	130.2 (9.2)	6	130.2 (9.2)
Arg-Gly/Gln-Gln	62	158.3 (6.1)	40	32.7 (1.1)
Arg-Gly/Gln-Glu	144	235.4 (4.1)	84	41.1 (0.9)
Arg-Gly/Glu-Glu	3	845.4 (715.6)	1	59.0
Gly-Gly/Gln-Gln	20	322.5 (32.0)	10	60.9 (7.4)
Gly-Gly/Gln-Glu	70	165.0 (5.7)	42	28.5 (0.9)
Gly-Gly/Glu-Glu	79	195.2 (5.4)	50	48.2 (1.3)
Total	441	207.6 (1.1)	265	39.8 (0.2)
Haplotype	All		≤ 200	
	n	Mean (SEM)	n	Mean (SEM)
AMP				
Arg-Arg/Gln-Gln	53	343.1 (0.1)	21	36.0 (2.6)
Arg-Arg/Gln-Glu	6	640.6 (0.3)	2	102.7 (13.7)
Arg-Gly/Gln-Gln	58	250.3 (0.1)	25	24.4 (1.7)
Arg-Gly/Gln-Glu	109	280.9 (0.1)	52	43.3 (1.1)
Arg-Gly/Glu-Glu	2	337.0 (0.7)	1	71.0
Gly-Gly/Gln-Gln	17	786.1 (0.1)	4	78.1 (8.2)
Gly-Gly/Gln-Glu	67	309.5 (0.1)	32	54.3 (1.6)
Gly-Gly/Glu-Glu	72	312.9 (0.1)	32	43.8 (1.6)
Total	384	312.5 (0.0)	169	41.8 (0.3)

Table 3. Fold difference and 95% confidence interval (CI) for difference for methacholine PD20 and adenosine monophosphate (AMP) PC20 between the β 2-adrenoceptor homozygous haplotypes

Methacholine		AMP	
Geometric mean fold difference	95% CI	Geometric mean fold difference	95% CI
Arg-Arg/Gln-Gln vs Gly-Gly/Gln-Gln	1.63	2.17	0.38–12.34
Arg-Arg/Gln-Gln vs Gly-Gly/Glu-Glu	1.29	1.22	0.50–2.98
Gly-Gly/Gln-Gln vs Gly-Gly/Glu-Glu	1.26	1.78	0.33–9.67

percentage predicted FEV₁ and atopic status with a regression equation of $\log \text{PC20} = 2.003 + 0.008 \times \text{percent predicted FEV}_1 - 0.111 \times \text{atopic status}$.

Discussion

We demonstrated no effect of haplotype in terms of BHR to methacholine or AMP in a population of asthmatic patients. Furthermore, the proportion of patients with each haplotype remained the same regardless of whether patients exhibited a clinically relevant degree of BHR to methacholine or AMP. Haplotype was not an independent predictor of hyper-responsiveness to either

methacholine or AMP. The significant but numerically small difference in FEV₁ between patients of a given haplotype was lost when corrected for the important cofactors namely steroid dose and atopic status.

The five most commonly occurring haplotype accounted for more than 80% of the patients. The most common haplotype was Arg–Gly/Gln–Glu (32%) followed by Gly–Gly/Glu–Glu (18%) and Gly–Gly/Gln–Glu (15%) with no patient having the Arg–Arg/Glu–Glu haplotype and only three patients having the Arg–Gly/Glu–Glu haplotype. The same three haplotypes were shown to be the most frequent in Caucasian asthmatic populations from the studies by Drysdale et al. (15) (Arg–Gly/Gln–Glu: 33%; Gly–Gly/Glu–Glu: 21%, Gly–Gly/Gln–Glu: 22%) and Lipworth et al. (16) (Arg–Gly/Gln–Glu: 26%, Gly–Gly/Glu–Glu: 29%, Gly–Gly/Gln–Glu: 15%). Furthermore, Drysdale et al. (15) also showed that the haplotype Arg–Arg/Glu–Glu occurred very rarely.

Previous authors have shown differences in BHR to methacholine in subjects depending on β 2-adrenoceptor polymorphism. D'Amato et al. (17) showed that the haplotype with a Gly at position 16 and Gln at 27 was associated with increased BHR with nearly 30% of subjects who were homozygous for this haplotype compared with 10% who were negative for this haplotype having a methacholine PD20 < 1600 μ g. Ramsay et al. (18) showed that homozygotes for Glu at 27 were less responsive to histamine and subjects with Gly at 16 were more likely to wheeze during a cold. However, both of these studies examined subjects who had not been screened for asthma, whereas, all of our patients presented with a physician diagnosis of asthma and were taking some form of asthma therapy. These studies, therefore, assess whether the genotype can be used to predict the presence of BHR in a healthy population rather than whether asthma severity is related to genotype. In this respect, Ramsay et al. (18) did not suggest that β 2-adrenoceptor polymorphism was predictive of developing asthma which is in keeping with the findings of other authors (19–21). Litonjua et al. (22) examined the effect of haplotype on BHR in case-control study of more than 500 subjects and showed that for nonsmokers, Gly16/Gln27 was negatively associated with BHR to methacholine. However, the control subjects were different from our patients as only 17% had a clinical diagnosis of asthma and they had a mean age of 63 years.

We cannot explain the discrepancy between our results and the findings of Fowler et al. (10) or Hall et al. (11) who showed increased BHR to methacholine with homozygous Gly 16 genotype and homozygous Gln 27 genotype, respectively. However, these studies employed a smaller number of patients, 158 and 65, respectively, and their results may have resulted from a type I error. In other words, the results could have occurred by chance. In another study with 630 adults with 40% having a physician or self-reported diagnosis of asthma, polymorphisms at either the positions 16 or 27 had no association

with BHR to methacholine (23). Other authors have shown that asthmatic patients with the Gly 16 polymorphism of the β 2-adrenoceptor are more likely to be receiving oral corticosteroids (21) or higher doses of inhaled corticosteroids (24) with no differences in emergency room visits, hospitalization or fatal asthma. These findings may be explained by differences in response to β 2 agonist therapy, with patients with Gly16 having less response and requiring higher doses of corticosteroid therapy, rather than Gly 16 predicting asthma severity or airway reactivity *per se*.

Although we have shown that β 2-adrenoceptor haplotype did not influence hyper-responsiveness to methacholine, there are data suggesting that different haplotypes of the β 2-adrenoceptor have an influence on bronchodilation and bronchoprotection with inhaled short- and long-acting β 2-agonists. Drysdale et al. (15), in a retrospective analysis, showed that change in FEV₁ with inhaled albuterol was significantly related to β 2-adrenoceptor haplotype with Gly–Gly/Glu–Glu having greater acute response in FEV₁ than Arg–Arg/Gln–Gln. In contrast, Lee et al. (25) showed the Gly–Gly/Glu–Glu haplotype to have significantly smaller changes in acute-systemic effects (serum potassium and diastolic blood-pressure responses) than the Arg–Arg/Gln–Gln haplotype following inhaled salbutamol, in a prospective study selecting these two homozygous haplotypes. Patients with homozygous Arg 16 genotype have a greater acute FEV₁ response inhaled short acting β 2-agonists in retrospective studies (26, 27). In the clinical setting when receiving regular albuterol, patients with the homozygous Gly 16 genotype had reduced morning peak expiratory flow (28), and more frequent major exacerbations than when on placebo treatment (29). In a retrospective pooled analysis of placebo-controlled studies with long-acting β -agonists, the Arg-16 polymorphism (as either homozygous or heterozygous genotype) was found to be associated with reduced protection against methacholine and AMP challenge after the first and last dose as compared with the homozygous Gly-16 genotype, with formoterol exhibiting greater subsensitivity of response than salmeterol (4).

We also showed that the β 2-receptor haplotype was not a significant independent predictor of hyper-responsiveness to methacholine or AMP bronchial challenge. In keeping with the findings of De Meer et al. (30), we showed that methacholine is determined by baseline FEV₁ whereas AMP is dependent on atopic status. This reflects the greater relationship between AMP hyper-responsiveness and airway inflammation (31).

This study was a retrospective analysis and, therefore, prone to the selection biases seen with this type of study. However, the number of patients involved in this analysis was sufficiently large for a marked differences in BHR between haplotype to be identified. As the confidence intervals for the difference between the homozygous haplotypes (Table 3) include a twofold difference, it is possible that differences in BHR to AMP or methacholine

may exist. However, the differences are likely to be small and would require a very large study to be detected. We investigated the haplotype of only two polymorphisms (codons at 16 and 27) as these are the most common. It may be that had our analysis included other polymorphisms differences in BHR could be detected.

In conclusion, we have shown that the degree of BHR to AMP and methacholine does not differ between β_2 -adrenoceptor haplotypes in patients presenting with a diagnosis of asthma. Although β_2 -adrenoceptor haplotypes do not predict airway reactivity, they may be important in predicting response to bronchodilator therapy.

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