

β_2 adrenoceptor promoter polymorphisms: extended haplotypes and functional effects in peripheral blood mononuclear cells

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Background: The β_2 adrenoceptor and its 5' untranslated region contain a number of genetic variants. The aim of this study was to investigate the potential for genetic variation at this locus to influence the expression of β_2 adrenoceptors on circulating peripheral blood mononuclear cells (PBMCs).

Methods: Genotype was determined in 96 individuals with asthma for four polymorphisms at the β_2 adrenoceptor locus. β_2 adrenoceptor binding and cyclic AMP responses to isoprenaline in PBMCs were determined and the relationship between genotype/haplotype and β_2 adrenoceptor expression and response to isoprenaline examined.

Results: β_2 adrenoceptor promoter polymorphisms were found to be common in white subjects. Strong linkage disequilibrium exists across this locus, resulting in the occurrence of several common haplotypes. No single polymorphism or haplotype was correlated with the level of β_2 adrenoceptor expression or cyclic AMP responses to isoprenaline *in vitro*.

Conclusion: β_2 adrenoceptor polymorphisms, when considered in isolation or by extended haplotypes, do not determine the basal level of expression or coupling of β_2 adrenoceptors in PBMCs from asthmatic subjects.

Beta₂ adrenoceptor agonists remain the mainstay bronchodilator agents used in the treatment of asthma. It has recently been suggested that some of the variability observed in response to these agents may be the result of genetic polymorphisms.^{1–4} The β_2 adrenoceptor locus on chromosome 5q31 contains a number of single nucleotide polymorphisms (SNPs). Within the coding region of the human β_2 adrenoceptor gene itself, nine SNPs have been identified,⁵ five of which are degenerate. Non-degenerate polymorphisms result in amino acid substitutions in codon 16 (Arg16→Gly), 27 (Gln27→Glu), 34 (Val34→Met), and 164 (Thr164→Ile). In recombinant and non-recombinant cell systems the Gly16 variant shows enhanced downregulation whereas the Glu27 variant is partially protected from downregulation.^{6,7} The rare Ile164 variant (allelic frequency ~2% in white populations) reduces the efficiency of receptor coupling with downstream effector pathways.⁸ *In vivo* there are data suggesting a functional role in fibroblast cell lines and cultured human airway smooth muscle for the polymorphisms at codon 16 and 164 and possibly also 27 (reviewed by Hall⁹).

Recent studies have also demonstrated the presence of a number of polymorphisms within the 5' untranslated region (UTR) of the human β_2 adrenoceptor gene. In a previous study¹⁰ we described eight SNPs within a 1.5 kb region upstream from the ATG start codon. This region is believed to be important for regulation of β_2 adrenoceptor gene transcription: it contains the majority of promoter activity for the human β_2 adrenoceptor gene and also includes a short open reading frame (sORF) for a 19 amino acid peptide known either as beta upstream peptide (BUP) or the β_2 adrenoceptor 5' leader cistron.¹¹ Using a reporter gene strategy we have shown that a construct containing the most frequently occurring non-wild type haplotype for the four SNPs contained within the 550 bp region of the 5'UTR had reduced luciferase expression in COS 7 cells compared with the wild type.¹² This 550 bp region contains the majority of promoter activity in the

5'UTR of the β_2 adrenoceptor gene. McGraw *et al* have also reported reduced β_2 adrenoceptor expression with the BUP Cys19→Arg polymorphism in a recombinant cell system where the β_2 adrenoceptor was expressed downstream of either the Cys19 or Arg19 form of BUP. More recent work, however, has suggested that when haplotypes—that is, a combination of polymorphisms across this region—rather than single polymorphisms are considered, the most common haplotype containing the Cys19 polymorphism is associated with higher levels of β_2 adrenoceptor expression in a recombinant cell system.¹³ In contrast, in a preliminary study in primary cultured human airway smooth muscle cells we were unable to detect significant effects of any of four 5'UTR SNPs on expression of firefly luciferase, either when each SNP was studied in isolation or in combination using the most frequently occurring haplotypes across this region.¹⁴ Hence, while functional data in recombinant cell systems suggest a potential role for the β_2 adrenoceptor 5'UTR polymorphisms, their importance to responses in subjects with or without asthma remains unclear.

In this study we concentrated on haplotypes and the two 5' SNPs most likely to be functionally important. The first of these SNPs results from a base change 47 bp upstream from the β adrenoceptor gene start codon (–47 T/C) which, as discussed above, substitutes an Arg for a Cys in BUP. The second 5'UTR SNP which appears potentially to be important results from a base change (T/C) at –367 bp from the start codon. This interrupts a putative Sp1 binding site in a region of the promoter containing strong positive promoter activity. To assess the potential relevance of these two 5'UTR polymorphisms and the known common polymorphisms in the coding region of the gene, this study had three aims: (1) to define the allelic frequencies of the 5'UTR polymorphisms in individuals with asthma; (2) to define the extent of linkage disequilibrium between these 5'UTR SNPs and those within the coding region and to determine the most common haplotypes in the white population; and (3) to assess the potential functional

effects of 5'UTR polymorphism on β_2 adrenoceptor expression in vivo by studying levels of β_2 adrenoceptor expression and cAMP responses to β_2 agonist in human peripheral blood mononuclear cells (PBMCs) in relation to genotype. Our hypothesis was that individuals carrying the -47 T/C variant, either in isolation or as part of an extended pro-downregulatory haplotype, would have lower resting levels of β_2 adrenoceptors on circulating PBMCs.

METHODS

Subjects

Two populations of asthmatic subjects were used for these studies from Dundee, UK and Groningen, the Netherlands.

Dundee

Fifty eight patients of mean (SD) age 35 (14) years with forced expiratory volume in 1 second (FEV₁) 2.58 (0.88) l (75.6 (17.9)% predicted) took part in the study; 48% were atopic. All 58 patients were taking inhaled corticosteroids; beclomethasone dipropionate at a mean dose of 522 (456) μ g per day in 32, budesonide at a mean dose of 967 (550) μ g per day in 18, and fluticasone propionate at a mean dose of 1500 (623) μ g per day in eight. Twenty were using long acting β_2 agonists (11 salmeterol, eight formoterol, and one bambuterol). All long acting β_2 agonist therapy was withdrawn for a washout period of at least 1 week before mononuclear cell tests (see below) were performed. Short acting β_2 agonists were withdrawn for at least 8 hours. No patients had received oral steroids for at least 3 months before the measurements. The study was approved by the trial ethics committee.

Groningen

The Dutch patients were taken from a randomised double blind parallel trial on the treatment of nocturnal asthma.¹⁵ Fifty patients entered the study and DNA was obtained from 38 individuals.¹⁵ The mean FEV₁ was 3.37 (0.10) l (86.9 (16.6)% predicted) and mean PC₂₀ methacholine was 1.78 mg/ml. Patients were included if they were non-smoking atopic asthmatics aged 18–45 years, reported a history of episodic dyspnoea or wheezing, with bronchial hyperresponsiveness to methacholine bromide (PC₂₀ <9.6 mg/ml). Inhaled corticosteroids were stopped 4 weeks before the study, oral corticosteroids 2 months before the study, and inhaled long acting β_2 agonists for 2 weeks. All data reported here were taken from the baseline measurement before the start of study medication. The genetic part of the study was approved by the medical ethics committee of the University Hospital Groningen and additional consent was obtained from all participants.

Isolation of peripheral blood mononuclear cells (PBMCs)

30–40 ml of blood was collected into tubes containing EDTA and diluted to 50 ml with phosphate buffered saline (PBS). Equal aliquots of diluted blood were then layered carefully onto 15 ml of Lymphoprep (Nycomed Pharma AS, Oslo, Norway) and centrifuged. A lymphocyte layer was then removed from each tube and combined before two further washes with PBS and centrifugation. The supernatant was discarded and the lymphocyte pellet resuspended in 5 ml PBS or Tris-buffer (Groningen). The preparation was counted to determine lymphocyte numbers and a sufficient volume was removed to obtain a total of 2.5×10^6 (Dundee) or $\times 10^7$ (Groningen) cells for measurement of cyclic AMP stimulation by isoprenaline. The remainder of the suspension was centrifuged, the supernatant discarded, and the lymphocyte pellet resuspended in the suspension buffer to give a concentration of 2.5×10^6 (Groningen) or 5×10^6 cells/ml.

Genotyping

Polymerase chain reaction (PCR) was used to amplify the β_2 adrenoceptor regions of interest. Table 1 shows the primers and conditions used for each locus. Coding region polymorphisms at codons 16 and 27 were genotyped using allele specific oligonucleotide (ASO) hybridisation as previously described.¹⁶ The -47 polymorphism within BUP was genotyped using a PCR based restriction length polymorphism (RFLP) assay. A *MspA1* I restriction site is present in the Arg19 (C) form which is not present in the Cys19 (T) sequence. The -367 polymorphism was also genotyped using RFLP; a *Bsu36* I site is present in the sequence containing the T allele while the C allele obliterates the recognition sequence of *Bsu36* I.

Measurement of cyclic AMP responses and β_2 adrenoceptor binding in PBMCs

Dundee

Lymphocyte β_2 adrenoceptor binding affinity (Kd) and maximum binding density (Bmax) were assayed on the prepared cell suspension (see above) after incubation in a waterbath at 37°C in tubes containing (-)¹²⁵I-iodocyanopindolol (ICYP) at eight concentrations from 5 to 160 pM. Half the tubes contained CGP 12177A HCl (1 μ M) to prevent ICYP binding to the receptor sites. After washing with assay buffer the bound and unbound suspension preparations were aspirated onto filter paper using a Brandel cell harvester and the resultant counts determined using a gamma counter. Specific receptor binding was calculated from total binding minus non-specific binding. Receptor density was calculated by Scatchard analysis using the specific and non-specific binding curves plotted for each concentration of ICYP. The intra-assay coefficient of

Table 1 PCR primers and conditions used to amplify the regions of interest for further analysis. The expected fragment lengths are also indicated

| Polymorphism | Primers | Conditions | Fragment length (bp) |
|------------------------|--|---------------------------|----------------------|
| Promoter -367 | Forward 5' CCTCTGCCTCGAGACCTCAAGCC 3' Reverse 5' CCGTCTGCAGCGCTCGAAC 3' | 60°C annealing; 30 cycles | 740 |
| Promoter -47 Arg19→Cys | Forward 5' CTTCGGGCTGCCGGCGTG 3' Reverse 5' GACATGGAAGCGGCCCTCAG 3' | 68°C annealing; 34 cycles | 1031 |
| Coding Arg16→Gly | Forward 5' CCCAGCCAGTGCCTTACCT 3' Reverse 5' CCGTCTGCAGCGCTCGAAC 3' | 60°C annealing; 36 cycles | 234 |

Table 2 Allele frequencies of the β₂ adrenoceptor polymorphisms -367 and -47 in the 5' flanking region, loci 16 and 27 in the coding region (n = 96)

| Locus | Allele 1 | Allele 2 |
|-----------|-------------|-------------|
| -367 T/C | T: 0.62 | C: 0.38 |
| -47 T/C | T: 0.60 | C: 0.40 |
| Arg16→Gly | Arg16: 0.31 | Gly16: 0.69 |
| Gln27→Glu | Gln27: 0.48 | Gln27: 0.52 |

variation for analytical imprecision was 5.8% for Kd and 10.3% for Bmax.

Cyclic AMP (cAMP) was determined as follows. The suspension containing 5 × 10⁶ cells was centrifuged and the pellet resuspended in PBS containing theophylline 100 μM and bovine serum albumin (10%). It was then stimulated with isoprenaline 10⁻⁴ M during the incubation at 37°C before terminating the reaction by heating to 95°C. After centrifugation the supernatant was removed and cAMP was determined by radioimmunoassay. The intra-assay coefficient of variation for analytical imprecision was 2.0%.

Groningen

Samples of 2.5 × 10⁶ cells in 900 μl Tris buffer containing 0.5 mM 3-isobutyl-1-methylxanthine to inhibit phosphodiesterase activity were preincubated for 10 minutes at 37°C. After preincubation, 100 μl isoprenaline (final concentration 1 μM) was added to duplicate samples. Basal cAMP levels were determined in duplicate by adding 100 μl buffer solution. For cAMP accumulation the samples were incubated for another 10 minutes at 37°C. The reaction was stopped by adding 100 μl 2 M HCl and 0.1 M EDTA, heated at 80°C for 10 minutes, centrifuged and the supernatants were collected and neutralised with CaCO₃.¹⁷ After removing excess CaCO₃ the cAMP concentration was measured by immunoassay (Biotrak, Amersham, UK).

The results from the two centres were pooled for analysis by genotype.

Statistical methods

Haplotype frequency estimation across the four loci was tested using the Estimated Haplotypes (EH) program developed by Ott and colleagues.^{18,19} Expected and observed frequencies were compared using log likelihood methods. Possible associations between genotypes/haplotypes and clinical phenotypes were tested using one way analysis of variance (ANOVA).

RESULTS

Allelic frequencies of the -367 T/C and -47 T/C β₂ adrenoceptor polymorphisms and linkage disequilibrium

Both the -47 T/C and -367 T/C SNPs were found to be common in the two groups of white subjects studied. The allelic frequencies of these polymorphisms are shown in table 2 and observed haplotype data are shown in table 3. It can be seen that the polymorphisms at -367 bp and -47 bp are in strong linkage disequilibrium and that linkage disequilibrium also exists between these polymorphisms and those at codons 16 and 27 within the coding region of the gene (table 4). Allelic frequencies were not significantly different in the two populations studied so the data were pooled for all subsequent analyses.

Effect of β₂ adrenoceptor promoter coding region haplotype on β₂ adrenoceptor expression and coupling in circulating PBMCs

In an attempt to define the potential functional effects of β₂ adrenoceptor polymorphism within the promoter region, we hypothesised that individuals with the Cys19→Arg polymorphism may show reduced levels of β₂ adrenoceptor expression in vivo. Levels of β₂ adrenoceptor expression, affinity, and function in PBMCs obtained from 58 Scottish asthmatic patients and 38 Dutch patients were therefore examined (table 5). These data show that, when either the BUP Cys19→Arg or the -367 T/C SNP are considered in isolation, no clear functional effects of these polymorphisms are evident. As would be predicted, no effect of the Arg16→Gly and Gln27→Glu β₂ adrenoceptor polymorphisms is apparent when considered in isolation (table 5).

Although levels of receptor expression measured by binding provide valuable information, it is possible that receptor coupling may be altered without an accompanying change in the

Table 3 Individual haplotypes observed in the asthmatic population for which all loci were genotyped (n = 93)

| -367 (T/C) | -47 (T/C) | Arg16→Gly | Gln27→Glu | No. | % Total |
|------------|-------------|-----------|-----------|-----|---------|
| C/C | C/C (Arg19) | Gly16 | Glu27 | 19 | 20 |
| T/T | T/T (Cys19) | Het | Gln27 | 10 | 11 |
| T/T | T/T (Cys19) | Arg16 | Gln27 | 9 | 10 |
| T/C | T/C (Het) | Het | Het | 8 | 9 |
| T/T | T/T (Cys19) | Het | Het | 8 | 9 |
| T/C | T/C (Het) | Gly16 | Het | 7 | 7 |
| T/T | T/C (Het) | Gly16 | Het | 4 | 4 |
| T/T | T/T (Cys19) | Gly16 | Glu27 | 3 | 3 |
| T/T | T/T (Cys19) | Gly16 | Het | 3 | 3 |
| T/C | T/C (Het) | Gly16 | Glu27 | 3 | 3 |
| C/C | C/C (Arg19) | Het | Het | 3 | 3 |
| T/T | T/C (Het) | Het | Het | 3 | 3 |
| T/C | T/T (Cys19) | Gly16 | Glu27 | 2 | 2 |
| T/C | T/C (Het) | Het | Gln27 | 2 | 2 |
| T/T | T/T (Cys19) | Gly16 | Gln27 | 2 | 2 |
| T/C | T/T (Cys19) | Het | Het | 2 | 2 |
| T/T | T/T (Cys19) | Arg16 | Het | 1 | 1 |
| T/T | C/C (Arg19) | Het | Gln27 | 1 | 1 |
| T/C | C/C (Arg19) | Gly16 | Het | 1 | 1 |
| T/T | T/C (Het) | Gly16 | Glu27 | 1 | 1 |
| T/C | T/T (Cys19) | Gly16 | Gln27 | 1 | 1 |

Table 4 Haplotype frequencies determined from the data in table 3 using the Estimated Haplotype program to estimate the haplotype combinations of the double, triple and quadruple heterozygous genotypes, both with and without association

| Haplotype | | | | Frequency | |
|------------|-----------|-----------|-----------|------------------|---------------------|
| -367 (T/C) | -47 (C/T) | Arg16→Gly | Gln27→Glu | With association | Without association |
| C | C (Arg19) | Gly16 | Glu27 | 0.32 | 0.06 |
| T | T (Cys19) | Arg16 | Gln27 | 0.26 | 0.05 |
| T | T (Cys19) | Gly16 | Gln27 | 0.16 | 0.12 |
| T | T (Cys19) | Gly16 | Glu27 | 0.13 | 0.13 |
| T | C (Arg19) | Gly16 | Glu27 | 0.036 | 0.091 |
| C | C (Arg19) | Arg16 | Gln27 | 0.029 | 0.023 |
| C | T (Cys19) | Gly16 | Glu27 | 0.022 | 0.079 |
| T | C (Arg19) | Gly16 | Gln27 | 0.017 | 0.089 |
| T | T (Cys19) | Arg16 | Glu27 | 0.007 | 0.058 |
| T | C (Arg19) | Arg16 | Gln27 | 0.007 | 0.038 |
| C | T (Cys19) | Gly16 | Gln27 | 0.007 | 0.075 |
| C | C (Arg19) | Gly16 | Gln27 | 0.00006 | 0.052 |

Log_e (I) χ^2 statistic = 249.08, df = 11 (p = 0.00008) suggests strong linkage disequilibrium across these loci. Four haplotypes did not occur with association.

Table 5 Mean (SE) β_2 adrenoceptor levels of receptor expression (Bmax), receptor affinity (Kd), and the ability of isoprenaline to stimulate cyclic AMP formation (Emax)

| Loci | Genotype | Bmax (fmol/mg) | p value | Kd (pmol) | p value | Emax (% above basal) | p value |
|-----------|----------|----------------|---------|-------------|---------|----------------------|---------|
| -367 T/C | T/T | 2.75 (0.32) | 0.27 | 21.5 (2.26) | 0.71 | 289 (27.7) | 0.01* |
| | T/C | 2.17 (0.17) | | 19.5 (1.80) | | 452 (57.3) | |
| | C/C | 2.43 (0.22) | | 21.3 (1.69) | | 327 (39.3) | |
| -47 T/C | T/T | 2.78 (0.32) | 0.27 | 20.5 (2.36) | 0.98 | 305 (28.4) | 0.17 |
| | C/T | 2.38 (0.19) | | 20.9 (1.89) | | 409 (57.8) | |
| | C/C | 2.27 (0.18) | | 20.4 (1.54) | | 331 (37.5) | |
| Arg16→Gly | Arg16 | 2.55 (0.22) | 0.73 | 25.2 (3.24) | 0.11 | 290 (71.6) | 0.68 |
| | HET | 2.54 (0.17) | | 21.3 (1.40) | | 363 (40.2) | |
| | Gly16 | 2.33 (0.24) | | 18.6 (1.65) | | 339 (31.5) | |
| Gln27→Glu | Gln27 | 2.48 (0.23) | 0.26 | 22.4 (1.96) | 0.54 | 329 (31.3) | 0.89 |
| | HET | 2.22 (0.17) | | 19.5 (1.73) | | 353 (31.9) | |
| | Glu27 | 2.73 (0.29) | | 20.5 (1.88) | | 352 (30.9) | |

The data were averaged by genotype groups to test for a particular genotype association with a clinical phenotype (ANOVA). Data were derived from a Dundee population (n = 58) and a Dutch population (n=38). *p<0.05.

overall level of receptor expression. We therefore examined the ability of PBMCs derived from individuals with different genotypes to generate cAMP in response to isoprenaline. No clear differences in the ability of cells expressing different forms of the β_2 adrenoceptor to generate cAMP was evident between genotypes (table 5), but we did observe a small, statistically significant, increase in cAMP responsiveness in those individuals heterozygous for the -367 T/C SNP. However, no trend was observed in homozygous individuals to support a role for this SNP and we believe this to represent a false positive result. In a post hoc subanalysis we also compared the cAMP responsiveness in those receiving inhaled steroid at the time of the study (the 58 subjects in the Dundee study) and those in whom treatment with inhaled steroids had been discontinued (the 38 Dutch patients). Again, no significant differences were observed between groups defined by genotype (data not shown).

Haplotype analysis of β_2 adrenoceptor expression in PBMCs

As the in vitro data suggested that effects of β_2 adrenoceptor promoter polymorphisms may only be apparent when relevant haplotypes are studied, we attempted to perform a haplotype analysis looking at levels of β_2 adrenoceptor expression and coupling in PBMCs studied ex vivo. To maximise the chance of

observing effects we only considered individuals homozygous for all the SNPs or those heterozygous at only a single position. This analysis was inevitably complicated by the strong linkage disequilibrium between these SNPs described above: no individuals with the potentially informative BUP Arg19 β_2 adrenoceptor Gly 16 Gln 27 homozygous haplotype were identified within our population (table 3). No significant difference was observed between levels of expression or cAMP responses to isoprenaline when considered by haplotype (fig 1), although the numbers are inevitably small for this analysis.

DISCUSSION

In this study a structured approach was taken to look for functional effects in vivo of recently described β_2 adrenoceptor promoter polymorphisms using PBMCs from patients with asthma. The main conclusions of the study are:

- The 5' UTR β_2 adrenoceptor promoter polymorphisms at -367 (T/C) and -47 (T/C) are common in the white population.
- Strong linkage disequilibrium exists across this region which results in the occurrence of several common haplotypes.
- No clear individual effect of β_2 adrenoceptor promoter polymorphisms can be seen on the level of expression or

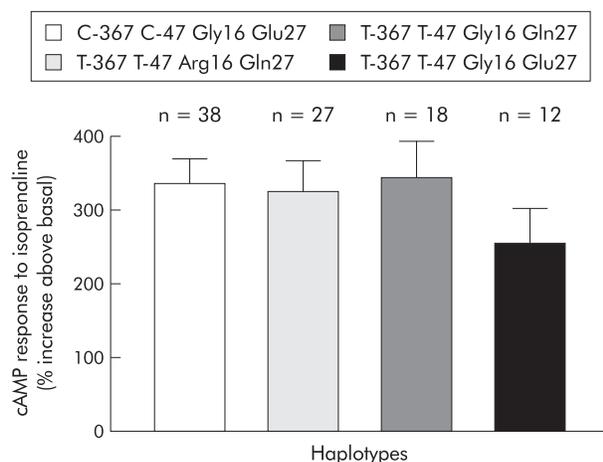


Figure 1 Percentage change in cAMP formation in response to isoprenaline stimulation of PBMCs with respect to haplotype.

coupling of β_2 adrenoceptors in PBMCs isolated from patients with asthma.

- No clear functional effect of any given haplotype on the level of receptor expression and coupling in PBMCs could be identified.

The promoter region for the human β_2 adrenoceptor gene (compared with other species such as the rat) has been relatively poorly studied. However, it is clear that promoter activity resides within a region of approximately 1.5 kb and that the majority of promoter activity is found in the first 550 bp upstream of the start codon.¹² This region contains a sORF for BUP which is believed to act as a translational (and possibly transcriptional) inhibition system.²⁰ Part of the mechanism whereby leader peptides such as BUP exert their effect is believed to depend upon the arginine content of these peptides: the Cys19→Arg polymorphism due to the SNP at -47 (T/C) increases the arginine content of the peptide from 3–4 residues out of the total of 19. This increased number of arginine residues would be predicted to increase its ability to exert translational inhibition. Data to support this suggestion have been published previously.¹¹ However, preliminary data reported by our group suggest that, at least with luciferase expression as an end point, in primary cultured airway cells the presence of the Cys19→Arg BUP polymorphism is inadequate by itself to cause functional effects.¹⁴ Given these conflicting data, the probable effects in vivo of these SNPs are unclear, and are further complicated by the strong linkage disequilibrium which exists across this region. Four SNPs have been described in the 550 bp region immediately upstream from the ATG which contains most of the promoter activity. However, the polymorphisms at -468 (C/G) and -20 (T/C) seem unlikely to contribute to functional effects, given that they do not alter known transcription factor binding sites. We therefore limited our analysis to the polymorphisms at -367 which is close to an SP1 site, -47 (Cys19→Arg in BUP), and the two common coding region polymorphisms at codons 16 and 27 of the β_2 adrenoceptor gene itself.

We then attempted to determine whether β_2 adrenoceptor expression ex vivo is determined by β_2 adrenoceptor promoter polymorphisms. The known functional effects of the codon 16 and 27 β_2 adrenoceptor polymorphisms are to alter downregulation profiles, both in recombinant cell systems⁶ and in primary cultured human airway smooth muscle.⁷ Given the in vitro data, we would predict that the -367 (T/C), Cys19→Arg (BUP), Arg16→Gly, Gln27→Glu β_2 adrenoceptor promoter coding region haplotype might lead to reduced in vivo expression of β_2 adrenoceptors without agonist exposure, which might increase following agonist exposure, as supported by

McGraw and coworkers.¹¹ We therefore investigated levels of β_2 adrenoceptor expression on circulating PBMCs isolated from patients with asthma. However, when considered in isolation, none of the above polymorphisms altered levels of receptor expression (Bmax) or receptor affinity (Kd). In addition, no difference in the ability of isoprenaline to drive cAMP formation in these cells was observed when different genotypes were considered in isolation. We did observe a small difference in individuals heterozygous for the -367 T/C SNP but, given that no effect was seen in the two relevant homozygous groups, this seems likely to be a false positive result. In a secondary analysis we also investigated whether there was evidence that inhaled steroid therapy (used in the Dundee subjects) may have masked any effect of genotype on cAMP responsiveness. No significant effects were apparent when only subjects without inhaled steroids were analysed, although the power of this analysis is obviously smaller than for the main study. Similarly, downregulation was not more apparent in subsets of the nocturnal asthma group defined by genotype or haplotype. Previously, we have shown that inhaled steroid therapy does not protect against β_2 adrenoceptor bronchodilator desensitisation,²¹ although effects have been observed on β_2 adrenoceptor mediated protection against bronchoconstriction.²²

One explanation for the lack of effect when each SNP is analysed in isolation is that functional differences are only present in individuals with a particular haplotype across this region. We were able to demonstrate strong linkage disequilibrium across this region, with the most frequently occurring haplotypes being -367 C, Arg19 BUP, Gly16, Glu27, and -367 T, Cys19 BUP, Arg16, and Gln27. We therefore examined the effects of different β_2 adrenoceptor haplotypes covering both the promoter and the functional polymorphisms within the coding region on β_2 adrenoceptor expression and coupling in PBMCs. Inevitably, this analysis was complicated by the lack of individuals with some potentially informative haplotypes; however, our data suggest that there is unlikely to be a marked effect of β_2 adrenoceptor promoter polymorphism on β_2 adrenoceptor expression in PBMCs. It is noteworthy that 42% of all the individuals studied in whom the haplotype could be determined carried one of two major haplotypes across this region. Hence, even if a given (rarer) haplotype was important in determining the level of expression and coupling of the β_2 adrenoceptor in PBMCs, very large population samples, or samples from ethnic groups in which the SNP frequencies are different, would be required to fully address this issue. While preparing this paper, a further study addressing this issue was published which showed strong linkage disequilibrium across this region in a USA population.¹³ This study also suggested that haplotypes rather than individual SNPs might predict bronchodilator reversibility, although the numbers were small.

In order to obtain the number of cells from a reasonable number of individuals necessary for this study we elected to use PBMCs as our assay system. Some studies have suggested that these may not be the best surrogate for studying β_2 adrenoceptor expression in the lung. However, desensitisation and/or downregulation has generally been easier to demonstrate in circulating PBMCs than in airway cells, thus it would seem unlikely that marked genotype/haplotype dependent effects would be seen in airway cells but not in PBMCs.

A preliminary assessment of the potential contribution of β_2 adrenoceptor promoter/coding region haplotypes to treatment response (defined as ΔFEV_1 to salbutamol) was able to show differences between groups defined by haplotypes, although the groups with the worst responses and best responses were not those that one would predict from in vitro functional studies.¹³ In a post hoc analysis we examined the degree of baseline reversibility in the subjects from Groningen for whom reversibility data and DNA were available (n=30, mean percentage reversibility 16.0%). No significant associations were seen when genotypes were analysed individually (n=30) or as haplotypes (n=13).

One possible criticism of this study is the use of two different patient populations. However, asthma severity was comparable between the groups gauged by FEV₁ and previous medication. No significant differences in genotype distribution were evident between the populations in the two centres, the genotype frequencies being in agreement with previous data on β_2 adrenoceptor polymorphism frequencies in other white populations. We therefore believe it unlikely that our results are influenced by the pooling of data obtained in Dundee and Groningen.

In summary, we have shown that β_2 adrenoceptor promoter polymorphisms are common in white subjects. Strong linkage disequilibrium exists between these promoter polymorphisms (and those within the coding region of the β_2 adrenoceptor), resulting in the occurrence of several common haplotypes. Using PBMC β_2 adrenoceptor expression and coupling as functional end points, we were unable to demonstrate marked functional consequences in vivo of any individual SNP or of the most common combinations studied using a haplotype approach. It is therefore unlikely that β_2 promoter polymorphisms play a major role in determining basal levels of β_2 adrenoceptor expression and coupling in vivo.

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