Genetics of asthma and atopy

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Chapter 11 ß2 adrenoceptor promoter polymorphisms: extended haplotypes and functional effects in peripheral blood mononuclear cells.

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Abstract:

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 4 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. This study demonstrated that β_2-adrenoceptor promoter polymorphisms are common in the Caucasian population. Strong linkage disequilibrium exists across this locus resulting in the occurrence of several common haplotypes. No single polymorphism nor haplotype was correlated with the level of β_2-adrenoceptor expression or cyclic AMP responses to isoprenaline in vitro.

Conclusion. We conclude that 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 obtained from asthmatic subjects.

Introduction

β_2-Adrenoceptor agonists remain the mainstay bronchodilator agents used in the treatment of asthma. Recently it has been suggested that some of the variability observed in response to these agents may be due to 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, 9 SNPs have been identified[5], 5 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 Ile164 variant whilst rare (allelic frequency ~2% in Caucasian 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 Hall[9]).

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 study[10] we described 8 SNPs within a 1.5kb 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 demonstrated that a construct containing the most frequently occurring non-wild type haplotype for the 4 SNPs contained within the 550bp region of the 5′ UTR showed reduced luciferase expression in COS 7 cells compared to wild-type.[12] This 550bp 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 (i.e. combination of polymorphisms across this region) rather than single polymorphisms are considered the commonest haplotype containing the Cys19 polymorphism actually 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, whilst 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 is due to a base change 47bp 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 is due to base change (T-C) at –367bp 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 we set out to achieve three aims. First, we wanted to define the allelic frequencies of the 5'UTR polymorphisms in individuals with asthma. Secondly, we wanted to define the extent of linkage disequilibrium between these 5' UTR SNPs and those within the coding region and determine the commonest haplotypes in the Caucasian population. Finally, in order to assess the potential functional effects of 5'UTR polymorphism on β_2-adrenoceptor expression in vivo, we studied levels of β_2-adrenoceptor expression and cAMP responses to β_2-agonist in human peripheral blood mononuclear cells in relation to genotype. Our hypotheses were 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 peripheral blood mononuclear cells.

Methods

Subjects

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

Dundee

A total of 58 patients from the Dundee centre were used, with mean (SD) age 35 (14) years, FEV_1 2.58 (0.88) litres, which was 75.6 (17.9) percent predicted. Forty-eight percent of subjects were atopic, all 58 patients were taking inhaled corticosteroid therapy, 32 with beclomethasone dipropionate at a mean dose 522 (456) µg per day, 18 budesonide at a mean dose of 967 (550) µg per day, and 8 with fluticasone propionate at a mean dose of 1500 (623) µg per day. 20 patients were using long-acting β_2-agonists, 11 with salmeterol, 8 with formoterol and 1 with bambuterol. All long-acting β_2-agonist therapy was withdrawn for a washout period of at least 1 week prior to measurement of lymphocytes β_2-adrenoceptor binding and isoproterenol stimulated cyclic AMP responses. Short-acting β_2-agonists were withdrawn for at least 8 hours. No patients had oral steroids for at least 3 months prior to 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 FEV_1 was 3.37 (0.104) litres (86.97 (16.6) percent predicted), and mean PC_{20} (methacholine) 1.78 mg/ml for these individuals. Patients were included if they were non-smoking, atopic asthmatic subjects aged 18 to 45 years, reported a history of episodic dyspnea or wheezing, and showed bronchial hyperresponsiveness to methacholine bromide (PC_{20} < 9.6 mg/ml). Inhaled corticosteroids were stopped 4 weeks prior to study, oral corticosteroids 2 months, and inhaled long-acting β_2-agonists for 2 weeks. All data shown in this paper were taken from the baseline measurement prior to 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

30-40mls of blood were collected into tubes containing EDTA and diluted to 50 ml with phosphate buffered saline. 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 2 further washes with phosphate buffered saline and centrifugation. The supernatant was discarded and the lymphocyte pellet resuspended in 5 mls of phosphate buffered saline or Tris-buffer (Groningen). The preparation was counted to determine lymphocyte numbers and a sufficient volume was removed in order to obtain a total of 2.5 x 10^6 (Dendee) or 10^7 cells (Groningen) cells which was required for cyclic AMP stimulation to isoprenaline. The remainder of the suspension was centrifuged and the supernatant discarded and the lymphocyte pellet resuspended in the suspension buffer to give a concentration of 2.5 x 10^6 (Groningen) or 5 x 10^6 cells per 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 loci. Coding region polymorphisms at codon 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 *Msp*A1 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 *Bsu*36 I site is present in the sequence containing the T allele while the C allele obliterates the recognition sequence of *Bsu*36 I.

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
Promoter -367	**Forward** **5'** CCTCTGCCTCGAGACCTCAAGCC **3'** **Reverse** **5'** CCGTCTGCAGACGCTCGAAC **3'**	60°C annealing & 30 cycles	740bp
Promoter -47 Arg19 → Cys	**Forward** **5'**CTTCGCGGCTGCCGGCGTG**3'** **Reverse** **5'** GACATGGAAGCGGCCCTCAG **3'**	68°C annealing & 34 cycles	1031bp
Coding Arg16 → Gly Coding Gln27 → Glu	**Forward** **5'**CCCAGCCAGTGCGCTTACCT **3'** **Reverse** **5'** CCGTCTGCAGACGCTCGAAC **3'**	60°C annealing & 36 cycles	234bp

Measurement of cyclic AMP responses and β_2-adrenoceptor binding in peripheral blood mononuclear cells

Dundee methods

Lymphocyte β_2-adrenoceptor binding affinity (Kd) maximum binding density (Bmax) were assayed on the prepared cell suspension (see above) after incubation in a 37°C waterbath in tubes containing (-)^{125} I-iodocyanopindolol (ICYP) at 8 concentrations from 5 to 160pM. Half the tubes contained CGP 12177A HCl (1mM) to prevent ICYP binding to the receptor sites. After washing with assay buffer the bound and unbound preparation of suspensions were aspirated onto filter paper using a Brandel cell harvester and the resultant counts determined by 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 variation for analytic imprecision was 5.8% for Kd and 10.3% for Bmax.

Cyclic AMP was determined for Dundee samples as follows. The suspension containing 5 x 10^6 cells was centrifuged and the pellet resuspended in phosphate buffered saline 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 cyclic AMP was determined by radioimmunoassay. The intra-assay coefficient of variation for analytical imprecision was 2.0%.

The methods used for samples from Groningen were similar. In brief, samples of 2.5 x 10^6 cells in 900ml Tris buffer containing 0.5 mM 3-isobutyl-1-methylxanthine to inhibit phosphodiesterase activity were preincubated for

10 minutes at 37°C. After the preincubation step, 100ml isoprenaline (final concentration 1mM) was added to duplicate samples. Basal cAMP levels were determined in duplicate by adding 100ml buffer solution. For cAMP accumulation the samples were incubated for another 10 minutes at 37°C. The reaction was stopped by adding 100 ml 2 M HC, 0.1 M EDTA, followed by heating at 80°C for 10 minutes. After centrifugation, the supernatants were collected and neutralised with $CaCO_3$.[17] After removing excess of $CaCO_3$, cAMP concentration was measured using an immunoassay (Biotrak, Amersham, UK).
Results obtained from each centre were pooled for the analysis by genotype.

Statistical methods

Haplotype frequency estimation across the 4 loci was tested, using the Estimated Haplotypes (EH) program 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 β_2-adrenoceptor polymorphisms and linkage disequilibrium

We found that both the -47 T-C and -367 T-C SNPs are common in the Caucasian populations studied. Data showing the allelic frequencies of these polymorphisms are shown in table 2 and observed haplotype data in table 3. It can be seen that the polymorphisms at bp –367 and –bp47 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). Allele frequencies were not significantly different in the two populations studied and hence data were pooled for all subsequent analyses.

Table 2 Allele frequencies of the β_2-AR polymorphisms, -367 and -47 in the 5' flanking region, loci 16 and 27 in the coding region (n = 96).

Loci	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

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 in β_2-adrenoceptor gene

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

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. The Ln (L) χ^2 statistic 249.08 d.f 11 (p = 0.00008) to suggest strong linkage disequilibrium across these loci. Four haplotypes did not occur with association.

Table 5 β_2-adrenoceptor levels of receptor expression (B max), receptor affinity (KD), and the ability of isoprenaline to stimulate cyclic AMP formation by β_2-adrenoceptor genotype

Polymorphism		Averages					
Loci	**Genotype**	**Bmax fmol/mg**	**ANOVA P values**	**KD pmol**	**ANOVA P values**	**Maximum response to Isoprenaline Emax (% above basal)**	**ANOVA P values**
-367 T/C	T/T	2.75 ± 0.32	0.27	21.5 ± 2.26	0.71	289 ± 27.69	0.01*
	T/C	2.17 ± 0.17		19.5 ± 1.80		452 ± 57.27	
	C/C	2.43 ± 0.22		21.3 ± 1.69		327 ± 39.30	
-47 T/C	T/T	2.78 ± 0.32	0.27	20.5 ± 2.36	0.98	305 ± 28.37	0.17
	C/T	2.38 ± 0.19		20.9 ± 1.89		409 ± 57.78	
	C/C	2.27 ± 0.18		20.4 ± 1.54		331 ± 37.55	
Arg16 → Gly	Arg16	2.55 ± 0.22	0.73	25.2 ± 3.24	0.11	290 ± 71.58	0.68
	HET	2.54 ± 0.17		21.3 ± 1.40		363 ± 40.23	
	Gly16	2.33 ± 0.24		18.6 ± 1.65		339 ± 31.48	
Gln27 → Glu	Gln27	2.48 ± 0.23	0.26	22.4 ± 1.96	0.54	329 ± 31.26	0.89
	HET	2.22 ± 0.17		19.5 ± 1.73		353 ± 31.89	
	Glu27	2.73 ± 0.29		20.5 ± 1.88		352 ± 30.95	

Mean and Standard Error, β_2-adrenoceptor levels of receptor expression (B max), receptor affinity (KD), and the ability of isoprenaline to stimulate cyclic AMP formation was averaged by genotype groups to test for a particular genotype association to a clinical phenotype (ANOVA). Data was derived from a Dundee population (n = 58) and a Dutch population (n=38) * $p<0.05$.

Effect of β_2-adrenoceptor promoter-coding region haplotype on β_2-adrenoceptor expression and coupling in circulating peripheral blood mononuclear cells

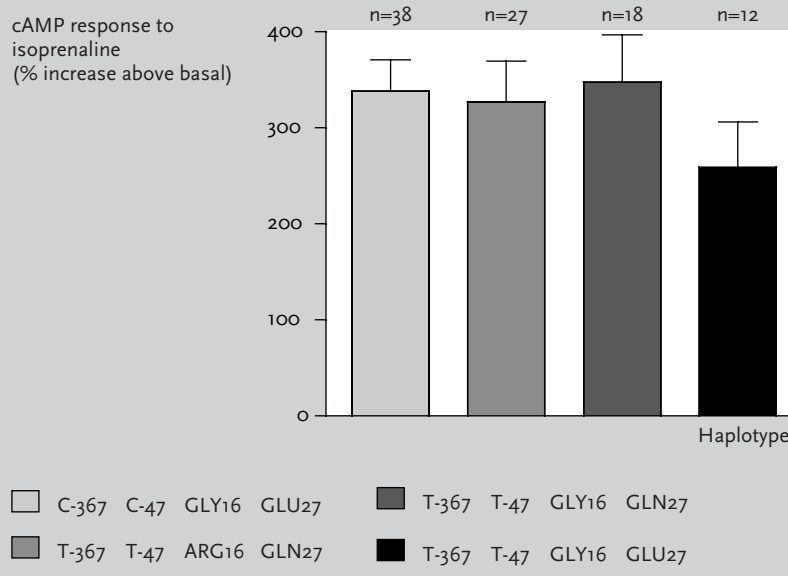
In order to attempt to define potential functional effects of β_2-adrenoceptor polymorphism within the promoter region, we hypothesised that individuals with the Cys19→Arg polymorphism may show reduced levels of β_2-adrenoceptor expression in vivo. We therefore looked at levels of β_2-adrenoceptor expression, affinity and function in peripheral blood mononuclear cells obtained from 58 Scottish asthmatics and 38 Dutch asthmatics (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 β_2-adrenoceptor polymorphisms is apparent when considered in isolation (table 5).

Although levels of receptor expression measured by binding provide valuable information, it remains possible that receptor coupling may be altered without an accompanying change in the overall level of receptor expression. Therefore, we examined the ability of PBMCs derived from individuals with different genotypes to generate cyclic AMP in response to isoprenaline. No clear differences in the ability of cells expressing different forms of the β_2-adrenoceptor to generate cAMP response was evident between genotypes (table 5). We did observe a small, statistically significant increase in cyclic AMP responsiveness in those individuals heterozygous for the –367T-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 posthoc sub-analysis we also compared the cyclic AMP responsiveness in those receiving inhaled steroid at the time of study (i.e. the 58 Dundee subjects) and those in which inhaled steroid therapy had been discontinued (i.e. the 38 Dutch individuals). Again, no significant differences were observed between groups defined by genotype (data not shown).

Haplotype analysis of β_2-adrenoceptor expression in peripheral blood mononuclear cells

Given our in vitro data suggesting 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 peripheral blood mononuclear cells 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 cyclic AMP responses to isoprenaline when considered by haplotype (Figure 1), although the numbers are inevitably small for this analysis.

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



Discussion

In this paper we describe a structured approach to look for functional effects in vivo of recently described β_2-adrenoceptor promoter polymorphisms using peripheral blood mononuclear cells from patients with asthma. The main conclusions of the study are that:

(i) The 5' UTR β_2-adrenoceptor promoter polymorphisms at –367 (T-C) and –47 (T-C) are common in the Caucasian population.
(ii) Strong linkage disequilibrium exists across this region which results in several common haplotypes occurring.
(iii) No clear individual effect of β_2-adrenoceptor promoter polymorphisms can be seen on the level of expression or coupling of β_2-adrenoceptors in peripheral blood mononuclear cells isolated from patients with asthma.
(iv) 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 (cf. other species such as the rat) has been relatively poorly studied. However, it is clear that promoter activity resides within a region of approximately 1.5kb and that the majority of promoter activity is found in the first 550 base pairs up-

stream 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 to 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 550bp region immediately upstream from the ATG which contains the majority of 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. Therefore, we 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 codon 16 and 27 of the β_2-adrenoceptor gene itself.

Next, we 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 systems 6 and in primary cultured human airway smooth muscle 7. However, 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 expression of β_2-adrenoceptors in vivo without agonist exposure which might increase following agonist exposure: this would also be predicted from McGraw et al's data. We attempted to study this by looking at levels of β_2-adrenoceptor expression on circulating peripheral blood mononuclear cells isolated from patients with asthma. However, when considered in isolation, none of the above polymorphisms altered levels of receptor expression (B max) or receptor affinity (KD). In addition, no difference in the ability of isoprenaline to drive cyclic AMP formation in these cells was observed when different genotypes were considered in isolation. We did observe a small difference in individuals heterozygous for the –367T-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 looked to see if there was evidence that inhaled steroid therapy (used in the Dundee subjects) may have masked any effect of genotype on cyclic AMP responsiveness. However, when analysed by subjects defined by inhaled steroid therapy, no

significant effects were apparent, although the power of this analysis is obviously smaller than for the main study. Similarly, down regulation 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, Arg19BUP, Gly16, Glu27 and –367 T, Cys19 BUP, Arg16, Gln27. We therefore examined the effects of different β_2-adrenoceptor haplotypes covering both the promoter and the functio nal 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 upon β_2-adrenoceptor expression at least in peripheral blood mononuclear cells. It is of note that 42% of all the individuals studied, where haplotype could be determined, carried one of 2 major haplotypes across this region. Hence, even if a given (rarer) haplotype were 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. Whilst preparing this manuscript a further study addressing this issue was published demonstrating strong lin kage 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 peripheral blood mononuclear cells 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, desensitization and/or downregulation has generally been easier to demonstrate in circulating PBMC than in airway cells, and hence it would seem unlikely that marked genotype/haplotype dependent effects would be seen in airway cells but not in PBMCs. It would be difficult to obtain pulmonary cells from adequate numbers of individuals with informative haplotypes to repeat this study using, for example, bronchoepithelial cell β_2-adrenoceptor expression as an appropriate endpoint.

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 percent 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 po pulations. However, asthma severity was comparable between the groups gauged by FEV_1 and previous medication. No significant differences in genotype distribution were evident between the populations in the two centres; the genotype frequencies being in keeping with previous data on β_2 adrenoceptor polymorphism frequencies in other caucasian populations. Therefore we 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 the Caucasian population. 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. However, 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 commonest combinations studied using a haplotype approach. It is unlikely therefore, 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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