β₂-Adrenoceptor polymorphism and bronchoprotective sensitivity with regular short- and long-acting β₂-agonist therapy

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ABSTRACT
The aim of the present study was to investigate bronchoprotective sensitivity in patients receiving regular treatment with short- and long-acting β₂-agonists and to evaluate any possible association with genetic polymorphism. Thirty-eight patients with stable mild to moderate asthma and receiving inhaled corticosteroids were randomized in a parallel group, double-blind, double-dummy fashion to receive 2 weeks of treatment with either formoterol (12 μg once daily, 6 μg twice daily or 24 μg twice daily) or terbutaline (500 μg four times daily). Bronchoprotection against methacholine challenge (as a provocative dose to produce a 20% fall in forced expiratory volume in 1.0 s: PD₂₀) was measured at baseline (unprotected) after an initial 1 week run-in without β₂-agonist, and at 1 h after the first and last doses of each treatment. The PD₂₀ values were log-transformed and calculated as change from baseline. Percentage desensitization of log PD₂₀ for first- versus last-dose bronchoprotection was calculated and analysed according to effects of treatment and β₂-adrenoceptor polymorphism at codon 16 or 27. The mean degree of desensitization for bronchoprotection was comparable with all four treatments and there were no significant differences in absolute PD₂₀ values after 2 weeks of chronic dosing. The PD₂₀ values were (as μg of methacholine, geometric means ± S.E.M.): formoterol, 12 μg once daily, 99 ± 42 μg; formoterol, 6 μg twice daily, 107 ± 44 μg; formoterol, 24 μg twice daily, 108 ± 45 μg; terbutaline, 500 μg four times daily, 88 ± 37 μg. All patients receiving formoterol, 24 μg twice daily, exhibited a loss of protection greater than 30% which was unrelated to polymorphism at codon 16 or 27. For codon 16, the use of lower doses of formoterol (12 μg once daily or 6 μg twice daily) showed wider variability in the propensity for protection loss in patients who were heterozygous, in contrast to a more uniform protection loss seen with homozygous glycine patients. The amount of protection loss was not significantly related to polymorphism at codon 16 or 27. The results of this preliminary study showed that bronchoprotective desensitization occurred readily in response to short- or long-acting β₂-agonist exposure irrespective of β₂-adrenoceptor polymorphism at codon 16 or 27. Further studies with larger patient numbers are required to further evaluate the effects of polymorphisms with lower doses of regular formoterol.

Key words: β₂-adrenoceptor, asthma, bronchial hyperreactivity, desensitization, formoterol, methacholine, polymorphism, terbutaline.
Abbreviations: FEV₁₀, forced expiratory volume in 1.0 s; PD, provocative dose.
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INTRODUCTION

β₂-Adrenoceptor agonists are recommended for first-line use as bronchodilator therapy in asthma [1,2]. Short- and long-acting β₂-agonists exhibit protective effects against a variety of direct and indirect bronchoconstrictor stimuli [3]. However, regular treatment with β₂-agonists is associated with tachyphylaxis to the functional antagonism against bronchoconstrictor stimuli [4]. There is evidence to show that inhaled corticosteroids and long-acting β₂-agonists given on a regular basis have additive effects in improving long-term asthma control [5–7]. This is now reflected in current asthma management guidelines in that addition of regular twice-daily long-acting β₂-agonist therapy is recommended for patients whose asthma is inadequately controlled on inhaled corticosteroid therapy [1,2].

Studies in vitro have shown that allelic polymorphisms of the β₂-adrenoceptor at codon 16 and 27 determine the degree of agonist-induced receptor down-regulation [8,9]. The glycine (Gly) polymorphism at codon 16 confers relative increased susceptibility to down-regulation compared with the arginine (Arg) polymorphism, whereas the glutamic acid (Glu) polymorphism at codon 27 confers relative resistance to down-regulation compared with the glutamine (Gln) polymorphism.

Regular treatment with inhaled formoterol in patients with asthma has been shown to produce a significantly greater degree of bronchodilator desensitization in patients with the homozygous Gly-16 genotype rather than the Arg-16 genotype [10]. Furthermore, it was found that the influence of the Gly-16 polymorphism dominated over any protective effects of the Glu-27 polymorphism. The Gly-16 polymorphism has also been shown to be associated with an attenuated bronchodilator response to inhaled salbutamol in children with asthma [11].

It is not known whether allelic polymorphisms at codon 16 or 27 of the β₂-adrenoceptor have a similar influence in terms of determining the propensity for tachyphylaxis to the bronchoprotective effects of β₂-agonists, and whether effects of long- and short-acting β₂-agonists might differ in this respect. The objective of the present study was therefore to evaluate the effects of regular treatment with formoterol and terbutaline in terms of their functional antagonism against methacholine-induced bronchoconstriction, and to evaluate whether there may be an association with genetic polymorphism.

METHODS

Patients

Thirty-eight patients with stable asthma of mild to moderate severity were randomized to completion, in which there was no prior knowledge of β₂-adrenoceptor genotype. Analysis of β₂-adrenoceptor polymorphisms was subsequently performed in all patients on a post-hoc basis at the end of study. All patients were required at entry to have a diagnosis of asthma according to America Thoracic Society criteria [12]. The patients were non-smokers, 18–45 years of age with a forced expiratory volume in 1 s (FEV₁) of at least 60 % of the predicted normal value. They were also required at screening to be responsive to methacholine challenge with a provocative dose of methacholine producing a 20 % fall in FEV₁ (PD₂₀) being equal to or less than 1000 µg, and exhibit at least a 2-doubling dose protection in response to a single test dose of 24 µg of formoterol.

All of the patients were receiving regular therapy with inhaled corticosteroids. The patients were using short-acting β₂-agonists on an occasional on-demand basis at a dose of less than four puffs per day. Long-acting β₂-agonists were being taken by seven patients and theophylline by four patients. All of the patients gave written informed consent for the study, which was approved by the Tayside Committee for Medical Research Ethics.

Study design

Patients were randomized into a parallel group, double-blind, double dummy design to receive treatment for 2 weeks with each of the following: (a) formoterol, 12 µg in the morning; (b) formoterol, 6 µg in the morning and at bed-time; (c) formoterol, 24 µg in the morning and at bed-time; (d) terbutaline, 500 µg in the morning, at midday, in the early evening and at bed-time.

Formoterol and terbutaline were both given by identical dry powder inhaler devices (Turbuhaler, Astra Draco, Sweden). In order to double-blind the randomized treatments, patients were also given an identical placebo Turbuhaler to use in addition to their active study inhaler, so that all of the patients were taking their study medication four times daily. Patients were instructed to take their study medication at the same time in the morning between 06.00 and 08.00 hours, at midday between 12.00 and 14.00 hours, in the early evening between 18.00 and 20.00 hours, and at bed-time at least 3 h after the early evening dose. All study medication was withheld for at least 12 h before the methacholine challenge at the end of each 2-week treatment period.

A baseline unprotected challenge was performed after an initial 1 week run-in, followed by a further run-in of 2–7 days before the first dose-protected challenge. All β₂-agonist therapy was withdrawn during the run-in period and substituted with an ipratropium bromide pressurized metered-dose inhaler in a dose of two puffs up to four times daily (as Atrovent Forte™, 40 µg per actuation, Boehringer Ingelheim, Bracknell, U.K.), which was used for rescue purposes. The same rescue medication with ipratropium bromide was also available to be used as required during each of the 2-week treatment periods.
Table 1  Demographic data by genotype

Values at screening are shown as arithmetic means ± S.E.M., except for PD\textsubscript{20} expressed as geometric means. There were no significant differences between polymorphisms at codon-16 or codon-27 for age, FEV\textsubscript{1.0}, PD\textsubscript{20} or steroid dose. Het = heterozygous.

<table>
<thead>
<tr>
<th></th>
<th>Gly-16 (n = 11)</th>
<th>Het-16 (n = 23)</th>
<th>Arg-16 (n = 4)</th>
<th>Glu-27 (n = 9)</th>
<th>Het-27 (n = 20)</th>
<th>Gln-27 (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>34 ± 3</td>
<td>38 ± 4</td>
<td>34 ± 8</td>
<td>34 ± 5</td>
<td>39 ± 3</td>
<td>33 ± 6</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>5/6</td>
<td>12/11</td>
<td>1/3</td>
<td>4/5</td>
<td>12/8</td>
<td>2/7</td>
</tr>
<tr>
<td>FEV\textsubscript{1.0} (% predicted)</td>
<td>85 ± 4</td>
<td>86 ± 4</td>
<td>91 ± 10</td>
<td>87 ± 5</td>
<td>84 ± 4</td>
<td>90 ± 6</td>
</tr>
<tr>
<td>Steroid (µg/day)</td>
<td>564 ± 118</td>
<td>878 ± 136</td>
<td>925 ± 364</td>
<td>433 ± 78</td>
<td>900 ± 141</td>
<td>733 ± 203</td>
</tr>
<tr>
<td>PD\textsubscript{20} (µg methacholine)</td>
<td>22 ± 8</td>
<td>42 ± 13</td>
<td>37 ± 36</td>
<td>23 ± 9</td>
<td>44 ± 15</td>
<td>30 ± 14</td>
</tr>
</tbody>
</table>

Table 2  Demographic data by treatment

Values at screening are shown as arithmetic means ± S.E.M., except for PD\textsubscript{20} expressed as geometric means. There were no significant differences between treatments for age, FEV\textsubscript{1.0}, PD\textsubscript{20} or steroid dose.

<table>
<thead>
<tr>
<th></th>
<th>Formoterol daily</th>
<th>Terbutaline times daily</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>39 ± 6</td>
<td>500 µg four times daily</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>5/3</td>
<td>4/5</td>
</tr>
<tr>
<td>FEV\textsubscript{1.0} (% predicted)</td>
<td>89 ± 5</td>
<td>84 ± 6</td>
</tr>
<tr>
<td>Steroid (µg/day)</td>
<td>950 ± 230</td>
<td>850 ± 206</td>
</tr>
<tr>
<td>PD\textsubscript{20} (µg methacholine)</td>
<td>28 ± 11</td>
<td>26 ± 13</td>
</tr>
</tbody>
</table>

The patients were instructed to withhold their rescue medication for at least 12 h before any of the methacholine challenges. The patients were requested to continue with their usual inhaled corticosteroid therapy throughout the run-in and treatment periods. All \( \beta\textsubscript{2} \)-agonist and theophylline therapy was withdrawn before the run-in.

Patients received written and verbal instructions on proper inhaler technique at each of the study visits. For this purpose a Turbuhaler inhalation training device was used (Astra Draco) as well as a metered-dose inhaler trainer device (Vitalograph, Buckingham, U.K.). Patients were also shown the correct use of a peak expiratory flow meter and were asked to plot the best of three morning and evening recordings on a diary chart.

Compliance was checked by evaluating the remaining doses in the study inhalers with each treatment and at least 75% compliance was required for data from a given treatment period to be considered evaluable. Patients were also requested to mark the time of taking each study drug on their diary card.

**Measurements**

\( \beta\textsubscript{2} \)-Adrenoceptor polymorphisms were identified as described previously [13]. In brief, genomic DNA was extracted from whole blood and a 234-bp fragment that spanned the regions of interest was generated by polymerase chain reaction. The genotype was determined by allele-specific oligonucleotide hybridization with probes homologous for the Arg-16, Gly-16, Gln-27 and Glu-27 forms of the receptor.

Methacholine challenges were performed unprotected at baseline after the initial run-in period, and protected challenges were carried out at 1 h after inhalation of the first and last doses of study medication. The procedure for the methacholine challenge protocol was previously validated and has been described elsewhere [14]. In brief, methacholine was administered in cumulative doubling doses from 3.125 µg to 6400 µg with a microprocessor-controlled dosimeter, at 5-min intervals until a 20% fall in FEV\textsubscript{1.0} was recorded. The PD\textsubscript{20} was determined by computer-assisted log-linear interpolation of the dose–response curve. Spirometry was measured according to American Thoracic Society criteria [15].

**Statistical analysis**

The study was powered at the 80% level in order to detect a 1.5 doubling dose difference in PD\textsubscript{20} between treatments with an inter-subject standard deviation of 1.3 doubling doses of methacholine. A significance level of \( P < 0.05 \) was used with a two-sided test. All of the values for PD\textsubscript{20} were logarithmically transformed and calculated.
as change from the initial unprotected baseline. The percentage difference in PD_{20} protection (as change from baseline) between the first and last doses of each treatment period was then calculated. Data for morning and evening peak flow values from diary cards were analysed by calculating the average values for the last 7 days of the run-in and the last 7 days of each treatment period. An overall statistical comparison was made by analysis of variance according to subject, treatment and genotype. This was followed by Bonferroni multiple-range testing so as to avoid multiple paired Student’s t-tests, in order not to confound the α error for the study.

RESULTS

The demographic data at entry are summarized in Tables 1 and 2 according to genetic polymorphisms and treatments respectively. All of the patients who were homozygous Glu-27 were also homozygous Gly-16, in keeping with the known linkage disequilibrium between these alleles. There were no significant differences between the polymorphisms or between the different treatments in terms of age, FEV_{1.0}, P D_{20} or inhaled corticosteroid dose. There were also no differences between the polymorphism groups in terms of previous use of long- or short-acting β_{2}-agonists.

The results for percentage desensitization between the first and last dose of each treatment are given in Tables 3 and 4 according to genotypes and treatments respectively. This showed that all treatments were associated with significant (P < 0.05) desensitization for loss of bronchoprotection between the first and last doses. Although formoterol, 24 μg twice daily, was associated with the highest value for mean percentage desensitization, this was not significantly different from the mean values for any of the other treatments. Absolute geometric mean PD_{20} values for all four treatments are depicted in Figure 1. There were no significant differences in absolute PD_{20} values after the last dose of each of the four treatments (geometric means ± S.E.M.): formoterol, 6 μg twice daily, 107 ± 44 μg; formoterol, 12 μg once daily, 99 ± 42 μg; formoterol, 24 μg twice daily, 108 ± 45 μg; terbutaline,

### Table 3  Methacholine bronchoprotection by genotype

<table>
<thead>
<tr>
<th>Gly-16</th>
<th>Het-16</th>
<th>Arg-16</th>
<th>Glu-27</th>
<th>Het-27</th>
<th>Gln-27</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n = 11)</td>
<td>(n = 23)</td>
<td>(n = 4)</td>
<td>(n = 9)</td>
<td>(n = 20)</td>
<td>(n = 9)</td>
</tr>
<tr>
<td>66 ± 11%</td>
<td>53 ± 8%</td>
<td>69 ± 18%</td>
<td>68 ± 12%</td>
<td>58 ± 8%</td>
<td>52 ± 12%</td>
</tr>
</tbody>
</table>

### Table 4  Methacholine bronchoprotection by treatment

<table>
<thead>
<tr>
<th>Formoterol</th>
<th>Terbutaline</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 μg twice daily</td>
<td>500 μg four times daily</td>
</tr>
<tr>
<td>12 μg once daily</td>
<td></td>
</tr>
<tr>
<td>24 μg twice daily</td>
<td></td>
</tr>
<tr>
<td>(n = 8)</td>
<td>(n = 9)</td>
</tr>
<tr>
<td>42 ± 12%</td>
<td>61 ± 12%</td>
</tr>
<tr>
<td>50 ± 11%</td>
<td></td>
</tr>
<tr>
<td>77 ± 11%</td>
<td></td>
</tr>
</tbody>
</table>
Polymorphism and bronchoprotection

Figure 2 Individual data for percentage protection loss (first versus last dose) according to genotypes at codon-16, broken down by treatments

A positive value for percentage protection loss indicates desensitization between the first and last dose. 1, formoterol, 6 µg twice daily; 2, formoterol, 12 µg once daily; 3, formoterol, 24 µg twice daily; 4, terbutaline, 500 µg four times daily.

Figure 3 Individual data for percentage protection loss (first versus last dose) according to genotypes at codon-27, broken down by treatments

A positive value for percentage protection loss indicates desensitization between the first and last dose. 1, formoterol, 6 µg twice daily; 2, formoterol, 12 µg once daily; 3, formoterol, 24 µg twice daily; 4, terbutaline, 500 µg four times daily.

500 µg four times daily, 88 ± 37 µg. When analysing mean values for percentage desensitization for pooled treatments, this revealed no significant difference in the propensity for desensitization between the different polymorphisms at either codon 16 or codon 27.

Individual values for percentage protection loss are depicted in Figures 2 and 3, according to genotypes by treatment. These individual data show that all patients with the homozygous Gly-16 genotype exhibited a protection loss greater than 30% in all four treatment groups. Furthermore, all patients receiving formoterol, 24 µg twice daily, showed a greater than 30% protection loss which was unrelated to polymorphism at codon 16 or 27.

Mean values for morning and evening peak expiratory flow during run-in and treatment periods are shown in
Table 5. There was a significant ($P < 0.05$) improvement in morning and evening peak expiratory flow only in association with treatment with formoterol, 24 μg twice daily, which was not influenced by polymorphism at codon 16 or 27.

**DISCUSSION**

The results of the present study showed that broncho-protective desensitization occurred with all four treatments and was not significantly related to β$_2$-adrenoceptor polymorphism. This was clearly evident on inspection of individual data for effects of formoterol, 24 μg twice daily, where a marked degree of protection loss occurred with all polymorphisms. Indeed, in three patients with the homozygous Arg-16 genotype, which is supposed to confer relative resistance to tachyphylaxis, there was a protection loss in excess of 70% in all cases with formoterol, 24 μg twice daily. The homozygous Glu-27 genotype, which is thought to protect against down-regulation, showed an identical pattern to that of the homozygous Gly-16, which is a reflection of the linkage disequilibrium between these polymorphisms [16]. Our results are in contrast to previous findings in terms of bronchodilator desensitization which occurs less readily than bronchoprotector desensitization, with the former being significantly associated with polymorphism at codon 16 of β$_2$-adrenoceptor [10]. *Ex-vivo* studies using lymphocyte β$_2$-adrenoceptors have also shown that the degree of down-regulation is associated with codon-16 polymorphism [17].

It was interesting to observe that the degree of protection loss was not influenced by the total daily dose of formoterol and was not significantly different when comparing twice daily formoterol and four times daily terbutaline. Our results also showed that even when using 12 μg of formoterol with a 24 h dosing interval, there was a comparable degree of protection loss to that with the same total daily dose of formoterol divided twice daily. This observation has also been reported when comparing once versus twice daily formoterol in terms of bronchoprotection loss against an indirect bronchoconstrictor challenge using adenosine monophosphate [18]. Desensitization has also been found when using salmeterol on a once daily basis in terms of protection loss against exercise challenge in children with asthma [19]. Taken together, these observations suggest that recovery of down-regulation does not occur with a 24 h dosing interval.

We accept that our study has limitations which are important to address. In particular, we acknowledge that the type 2 error was confounded by comparing the six different genotypes. Firstly, we only performed a retrospective genotype analysis at the end of the study and so it is perhaps not surprising that we had only four patients with the homozygous Arg-16 genotype, in keeping with the known prevalence of this polymorphism in the general population [13,16]. Three of these patients were randomized to receive the highest dose of formoterol (24 μg twice daily), which was associated with marked protection loss in all of these cases.

As none of the patients with the homozygous Arg-16 genotype received formoterol, 12 μg once daily or 6 μg twice daily, it is possible that an effect of polymorphisms at codon 16 may have been missed in association with lower doses. It was also evident at codon 16 with lower doses of formoterol that there was considerable variability in the degree of protection loss in heterozygous patients, in contrast to a more uniform protection loss in all homozygous glycine patients. The small number of subjects with a particular genotype precluded any subgroup analysis within a treatment group.

It is also worth pointing out that we analysed the overall effects of polymorphism irrespective of different treatments, although there were no significant differences between the four treatment groups in terms of the...
propensity for agonist-induced desensitization. This was evident in that the absolute PD_{50} values for all four treatments were similar after 2 weeks of chronic dosing. The comparable degree of protection loss with the different formoterol regimes may reflect its high level of \( \beta_2 \)-adrenoceptor intrinsic activity, and hence a greater propensity for inducing down-regulation. This may also have been compounded by the high level of lung deposition associated with the Turbuhaler delivery system [20]. We elected to use patients who were already receiving inhaled corticosteroids as current asthma management guidelines suggest that regular long-acting \( \beta_2 \)-agonists should only be used in this way as combination therapy [1,2]. The occurrence of significant bronchoprotective loss despite concomitant inhaled corticosteroid therapy is in keeping with previous studies also using the methacholine challenge model [21–23]. It is pertinent to consider our results in the context of large clinical studies where the use of regular formoterol with inhaled corticosteroid was associated with improvements in disease control and exacerbation rates [7].

In conclusion, our preliminary study showed that desensitization occurred to the bronchoprotective effects of regular treatment with short- or long-acting \( \beta_2 \)-agonist therapy, which was not determined by genetic polymorphism of the \( \beta_2 \)-adrenoceptor. Further studies with larger patient numbers for each genotype are required to evaluate whether polymorphism determines the level of protection loss with lower doses of formoterol.

ACKNOWLEDGMENTS

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