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Variable Length Poly-C Tract Polymorphisms of the β_2 -adrenergic Receptor 3'UTR Alter Expression and Agonist Regulation

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Abstract

β_2 -adrenergic receptors (β_2 AR) expressed on airway epithelial and smooth muscle cells regulate mucociliary clearance and relaxation, and are the targets for β -agonists in the treatment of obstructive lung disease. However, the clinical responses display extensive interindividual variability, which is not adequately explained by genetic variability in the 5'-flanking or coding region of the intronless β_2 AR gene. The nonsynonymous coding polymorphism most often associated with a bronchodilator phenotype (Arg16) is found within three haplotypes that differ by the number of C's (11, 12 or 13) within a 3'UTR poly-C tract. To examine potential effects of this variability on receptor expression, BEAS-2B cells were transfected with constructs containing the β_2 AR (Arg16) coding sequence followed by its 3'UTR with the various polymorphic poly-C tracts. β_2 Arg16-11C had 25% lower mRNA expression and 33% lower β_2 AR protein expression compared to the other two haplotypes. Consistent with this lower steady-state expression, β_2 Arg16-11C mRNA displayed more rapid and extensive degradation after actinomycin D treatment compared to β_2 Arg16-12C and 13C. However, β_2 Arg16-12C underwent 50% less downregulation of receptor expression during β -agonist exposure compared to the other two haplotypes. Thus, these haplotypes direct a potential low-response phenotype due to decreased steady-state receptor expression combined with wild-type agonist-promoted downregulation (β_2 Arg16-11C), and, a high-response phenotype due to increased baseline expression combined with decreased agonist-promoted downregulation (β_2 Arg16-12C). This heterogeneity may contribute to the variability of clinical responses to β -agonist, and genotyping to identify these 3'UTR polymorphisms may improve predictive power within the context of β_2 AR haplotypes in pharmacogenetic studies.

Keywords

Polymorphism; adrenergic receptor; asthma

INTRODUCTION

The β_2 -adrenergic receptor (β_2 AR) plays a key role in regulating a number of cellular processes in the lung (2, 3, 9). Of particular therapeutic interest is the localization of these receptors on airway epithelial cells, where they modulate fluid and electrolytic flux and ciliary beat frequency, and on airway smooth muscle where they act to relax constricted airways. These actions have led to the common use of β_2 AR agonists for prevention, and acute treatment of, obstructive lung diseases such as asthma and chronic obstructive lung disease. The β_2 AR gene has been found to be polymorphic in the coding region (17), with two common nonsynonymous polymorphic sites at amino acid positions 16 and 27. At position 16, Arg or Gly can be present, and at position 27 Glu or Gln. Based on in vitro data from transfections into a fibroblast cell line with constructs consisting only of the respective open-reading frames, certain phenotypes were assigned to these polymorphisms (8). These results led to a number of clinical studies in asthma, examining the potential for variants at either the 16 or 27 positions to influence the long-term response to regular β -agonist use. A number of studies (14, 15, 22, 30, 33), each of somewhat different design but nevertheless examining well-accepted outcomes, have revealed that patients with the Arg16 genotype display a tachyphylaxis-like phenotype, with physiologic and/or clinical deterioration during long-term use of β -agonists. The molecular basis of this genotype-phenotype correlation is not entirely clear (12, 19). Furthermore, within the group of subjects genotyped as “Arg16”, there is nevertheless phenotypic variability in these clinical outcomes. This suggests that heterogeneity within the β_2 AR gene apart from this locus may be influencing phenotype among patients categorized as Arg16 from single-site genotyping at this locus. Such unrecognized genetic variability could also explain the discrepant results in the published literature regarding associations with the Arg16 genotype during β -agonist treatment. Indeed, several recent studies have revealed no association between the Arg16 genotype and bronchodilator-related phenotypes (4, 11, 32). We have recently found (13) that Arg16 is partitioned into multiple haplotypes due to polymorphisms of a poly-C repeat region of the β_2 AR 3′ untranslated region (3′ UTR). Indeed, Caucasians with the Arg16 genotype represent three different haplotypes defined by the 3′ UTR 11, 12 or 3 C’s, with haplotype frequencies within the Arg16 group ranging from ~14–43% (13). Given that the 3′ UTR has been implicated in mRNA stability and translation, we sought to ascertain whether this naturally occurring variability in this region, within the context of Arg16, can alter expression or regulation of the β_2 AR. Studies were carried out with transfections of the human airway epithelial cell line BEAS-2B. Constructs consisted of contiguous sequence representing the β_2 AR open reading frame (with Arg at amino acid 16) with its 3′ UTR. Other coding polymorphisms and the poly-C tract consisting of 11, 12 or 13 C’s were in phase as they appear in the three common haplotypes. We show that this variation in the poly -C region alters mRNA stability and thus affects steady-state cell surface expression of the β_2 AR protein. In addition, the extent of agonist-promoted downregulation is dependent on the number of C’s in this region. Taken together, these results provide evidence for the importance of the 3′ UTR poly-C variants, and the potential need for including these genotypes in addition to other SNPs within the gene in association studies involving β_2 AR-related phenotypes.

MATERIALS AND METHODS

Constructs

The bacterial artificial chromosome clone RP11-44B19 was utilized as a template to create the various polymorphic forms of the β_2 AR. In keeping with prior literature (13, 23) the A of the initiator ATG of the coding block is designated as nucleotide 1. The constructs consisted of the open reading frame of the intronless β_2 AR followed by its 3'UTR through the polyadenylation sequence. The nucleotides at positions 46 and 79, within the codon for amino acids 16 and 27 were A and C, representing Arg and Gln, respectively. The nucleotides at the sites where coding block synonymous polymorphisms occur (252, 491, 523, 1053 and 1239) were as they appear in haplotypes bearing the 46 and 79 nonsynonymous polymorphisms as well as the polymorphisms in the poly-C tract. The variable length poly-C tract can be considered to begin at nucleotide 1266 and commonly extends to 1278, but rarely (14 C's) to 1279 (Table 1). Site-directed mutagenesis was utilized to alter the number of C's within this region resulting in a tract of 11, 12, or 13 C's as is observed in the human population within the Arg16 haplotype (also referred to as haplotype 4 (7)). The final expression constructs consisted of the β_2 AR open reading frame with the indicated 3'UTR in the vector pcDNA3.1, which were sequenced for verification.

Cell culture and transfection

BEAS-2B cells were obtained from American Type Culture Collection (Manassas, VA) and maintained as described in Ham's F12 medium with 10% bovine calf serum (21). Transfections with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) were carried out as previously described (26). Briefly, $\sim 10^7$ cells in monolayers in a T75 flask were treated with 5.0 μ g β_2 AR DNA and 10 μ l of the Lipofectamine reagent in 8 ml of serum free media for 6 hrs. Subsequently, 8 ml of additional media with serum to provide a concentration of 10% was added and the cells incubated overnight. The next day the media was changed or the cells were split and plated into smaller dishes. Cells were harvested for the various experiments, as described below, 48 hrs after the addition of the DNA and transfection reagent. Initial studies showed that this method and concentration of DNA resulted in β_2 AR expression ~ 5 – 7 fold over endogenous expression in BEAS-2B cells, and was within the linear portion of the transfected DNA:expression curve. To assess β_2 AR mRNA stability, cells were transfected as above, and after 48 hours treated with 5 μ g/ml actinomycin D in serum-free media for the indicated times. For agonist-promoted downregulation studies, 48 hours after transfection, companion dishes of cells in serum-free media were treated with 100 μ M ascorbic acid, or ascorbic acid with 10 μ M isoproterenol, and the cells harvested 12 hrs later.

Radioligand binding

BEAS-2B cells endogenously express β ARs which are $\sim 95\%$ of the β_2 AR subtype (1), and the transfected receptors are also β_2 AR, overexpressed by ~ 5 – 7 -fold over the endogenous background. Thus receptor expression, as quantitated using the high-affinity β AR radioligand 125 I-cyanopindolol (125 I-CYP, Perkin-Elmer, Waltham, MA), was considered all of the β_2 AR subtype, given the $<1\%$ β_1 AR subtype present under these conditions. Transfected cells in monolayers were washed three times with PBS, and one-half of the plate

was scraped in PBS with a rubber policeman to detach the cells, which were transferred to a centrifuge tube. Cells were lysed by addition of 5 mM Tris, 2 mM EDTA pH 7.40 and sheared by repeated pipetting. The preparation was centrifuged at $30,000 \times g$ for 15 min and the pellet resuspended in 75 mM Tris, 12 mM $MgCl_2$ and 2 mM EDTA, pH 7.40. Radioligand binding with ^{125}I -CYP was performed in triplicate as previously described (10), with propranolol (10 μM) used to define nonspecific binding. Incubations were carried out at 25°C for 2 hrs. Specific binding was normalized to protein and expressed as fmol/mg protein.

Quantitative RT-PCR

The remaining one-half of the flask of monolayered cells was treated with TRIzol reagent (Invitrogen, Carlsbad, CA) and frozen at $-80^\circ C$, for batch extraction of RNA, and DNase 1 treatment, as described previously (27). Reverse transcription reactions were carried out with Moloney murine leukemia virus reverse transcriptase (MultiScribe, Applied Biosystems, Foster City, CA) and 500 ng of extracted RNA. Reactions were incubated at 25°C for 10 min, 37°C for 120 min, and 65°C for 5 sec and then cooled to 4°C. Real time PCR was carried out using 1/50 of the reverse transcription reaction. The TaqMan (Applied Biosystems, Foster City, CA) probe and primer sets were from Applied Biosystems for the human β_2AR , which provide an amplicon of 66 bp representing nucleotides 500–565 of the β_2AR coding region. Similar reagents from Applied Biosystems were used to amplify GAPDH. C_T values were quantitatively compared to $\beta_2Arg16-12C$, or time 0 values as indicated, using the “ $2^{(-C_T)}$ ” method (20, 25).

Statistical analysis

Data are presented as mean $\pm$ SE. Data from radioligand or quantitative RT-PCR studies were compared by the use of paired t-tests of the raw data. For presentation purposes the results are shown normalized to the $\beta_2Arg16-12C$ haplotype, so as to illustrate the relationship between haplotypes. Time course data were analyzed by ANOVA to reveal overall differences between curves, and the data were also fit by iterative techniques to a one-phase exponential decay to determine kinetic parameters (Prism, GraphPad, San Diego, CA). Significance was considered when $P < 0.05$.

RESULTS

Shown in Fig. 1 are the polymorphisms of the β_2AR open-reading frame and 3'UTR. Table 1 shows the relevant nucleotides within the three Arg16-containing haplotypes chosen for study based on the variable number of C's within the poly -C region. These are designated as $\beta_2Arg16-11C$, $\beta_2Arg16-12C$, and $\beta_2Arg16-13C$. (The gap shown at position 1269 is present to maintain consistency in the numbering system, since very rarely 14C's in the tract have been noted (13).) BEAS-2B cells were transfected with constructs representing each of the three haplotypes. These cells were chosen because they are of human origin, are a relevant cell-type in asthma, and have a background level of β_2AR expression amounting to 197 ± 13 fmol/mg (N=6). This latter quality implies that they have the necessary factors for regulation of receptor expression, thus potentially representing a better model-cell compared to those cells lacking endogenous β_2AR expression. The BEAS-2B cells transfected with the

β_2 AR-12C haplotype expressed at an average level of $1,507 \pm 180$ fmol/mg (N=6), providing a sufficient level over background (5–7-fold) to compare the effects of the poly-C tract polymorphisms. The β_2 AR haplotype expression constructs, representing contiguous sequence from the open reading frame to a distal 3'UTR polyadenylation sequence, were transfected into cells in parallel, and one-half of the culture plate was harvested for protein and the other for RNA 48 hours after transfection (a time point when steady-state protein and mRNA levels are achieved, data not shown). β_2 AR protein expression was determined by quantitative radioligand binding using 125 I-CYP. β_2 AR mRNA levels were determined by quantitative RT-PCR, with GAPDH mRNA utilized as a control. The mean results of protein and mRNA expression by Arg16-poly-C haplotypes are shown in Fig. 2. β_2 Arg16-12C was selected as the reference haplotype, and β_2 AR protein and mRNA expression for each of the other haplotypes was normalized to β_2 Arg16-12C values for each individual experiment. As shown in Fig. 2A, 12 or 13C's had equivalent β_2 AR protein expression, while 11C's resulted in decreased expression ($75 \pm 4.7\%$ of β_2 Arg16-12C, $P < 0.01$). This pattern was also observed with mRNA expression, with β_2 Arg16-11C having lower expression than the other two haplotypes ($66 \pm 10\%$ of β_2 Arg16-12C, $P < 0.01$). Given the agreement between β_2 AR protein and mRNA expression results by haplotype, and the fact that the open reading frames of the three do not differ in their sequences, we considered that the basis for the altered expression of β_2 Arg16-11C was due to decreased mRNA stability imposed by the 3'UTR polymorphism. To address this, multiple plates of cells were treated with actinomycin D to block transcription 48 hours after the transfection; β_2 AR mRNA half-life ($t_{1/2}$) was determined by quantitating mRNA by quantitative RT-PCR at various timepoints up to six hours after actinomycin D treatment. The maximal extent of mRNA loss over 6 hours was greatest for β_2 Arg16-11C, being $55 \pm 4\%$ compared to $39 \pm 5\%$ and $44 \pm 3\%$ for β_2 Arg16-13C and β_2 Arg16-12C, respectively (Fig. 3, $P < 0.05$). The $t_{1/2}$ was lower for β_2 Arg16-11C (0.62 ± 0.06 , $P < 0.05$) compared to those for the -13C and -12C variants (1.6 ± 0.43 and 0.98 ± 0.16) which were not different from each other. These data are consistent, in direction and magnitude, to the steady-state expression phenotypes shown in Fig. 2, where β_2 Arg16-11C has lower β_2 AR mRNA and protein expression compared to the other two receptors.

We next considered whether agonist-promoted downregulation of β_2 AR was affected by the poly-C tract. β_2 AR expression is regulated by long-term agonist exposure by alterations in mRNA transcription or degradation, translation, internalization of receptors, and degradation of β_2 AR protein (9). Thus for these assays expression was quantitated with 125 I-CYP binding, which represents the final expression of the mature receptor at the cell membrane, taking into account all the above processes. The results from these experiments, carried out with exposure of the cells to $10 \mu\text{M}$ of the β AR agonist isoproterenol for 12 hours, are shown in Fig. 4. Both β_2 Arg16-11C and -13C underwent equivalent downregulation amounting to a decrease of 21 ± 5.2 and $26 \pm 5.3\%$, respectively ($P > 0.10$). In contrast, β_2 Arg16-12C underwent less agonist-promoted downregulation ($14 \pm 5.4\%$, $P < 0.05$ vs. either of the other two haplotypes).

DISCUSSION

As introduced earlier, the Arg16 polymorphism of the β_2 AR has been associated with various β -agonist phenotypes in asthmatics (14, 15, 22, 30, 33). However, discrepancies in these findings from other studies is readily appreciated (4, 11, 32). Of interest has been efforts at refinement of the genetic basis of these phenotypes, either by expanding genotyping to cover additional polymorphisms in the gene (7, 13), or considering other genes in the β_2 AR signaling pathway (29). One justification for the use of the polymorphism at position 16 within the β_2 AR coding region has been that it defines a major haplotype, if one considers the 5'-flanking and coding polymorphisms. This haplotype, as describe by Drysdale et al. (7), is denoted haplotype 4. However, we now know that Arg16 genes are partitioned into haplotype groups based on 11, 12, or 13 C's in the 3'UTR (13). Considering the 5'-flanking and coding nonsynonymous polymorphisms, Arg16/haplotype 4 Caucasians differ *only* at this poly-C region, with the following distribution (%): 11C $\approx$ 14%, 12C $\approx$ 41% and 13C $\approx$ 43%. We thus explored the effects of these variations within the context of Arg16, using transient transfections of airway epithelial cells, with measurements of mRNA and protein expression under various conditions. We found that steady-state expression of β_2 Arg16-11C was decreased by 25% (protein expression) and 33% (mRNA expression) compared to β_2 Arg16-12C and -13C. When cells were treated with actinomycin to block mRNA transcription, β_2 Arg16-11C mRNA underwent substantially greater, and more rapid, degradation compared to the other two poly-C haplotypes. These data indicate that in the basal state, β_2 Arg16-11C has lower expression due to a relatively unstable mRNA leading to lower steady-state levels of transcript and protein expression. This implies that patients with this specific haplotype may have decreased bronchodilator responses to inhaled β -agonist compared to other Arg16-containing β_2 AR haplotypes. It is interesting to note that there has been reported a substantial variability in the *in vivo* β -agonist response in individuals with the homozygous Arg16-based 5'/coding haplotype (FEV₁ % predicted equal 8.53 with a standard deviation of 6.66 in Caucasians with haplotype 4) (7). This *in vivo* variability within the Arg16 haplotype group could be due to additional variability within this haplotype, represented by the variable number of C's in the 3'UTR.

We also examined the effect of persistent β -agonist exposure on receptor downregulation between the variable C haplotypes. Agonist-promoted β_2 AR downregulation leads to a tachyphylaxis-like phenotype in patients receiving repetitive β -agonists on a chronic basis (18). We find that the low expressing β_2 Arg16-11C undergoes downregulation similar to the 13C haplotype. So potentially, individuals with the Arg16-11C haplotype may have a decreased initial response to β -agonists, and display tachyphylaxis such that their ultimate responsiveness is quite low. Interestingly, the 12C variant which has wild-type baseline expression, undergoes less downregulation compared to the other two haplotypes. This may represent the most favorable of the Arg16 haplotypes, since the initial response to β -agonist is predicted to be unimpaired and little tachyphylaxis would be expected. The poly-C region within the β_2 AR 3'UTR lies close to ($\sim$ 20 bp downstream) an AU-rich region, which is known to affect the rate of mRNA translation of β_2 AR mRNA (16, 31) and mRNA stability of several other genes (5, 6, 24). Furthermore, this poly-C region is localized within a predicted hairpin structure within the 3'UTR, with the stem formed by nucleotides within

the poly-C tract. 3'UTR hairpins have variable effects on mRNA processing (28). Whether these relatively subtle changes (11, 12 or 13 C's) have an effect on the AU-region, or the hairpin structure, is not predictable from current modeling techniques. In the model cell system utilized, we felt it was essential that the cells be of human origin, are a cell-type of importance to asthma, and indeed express the β_2 AR. The transfected β_2 AR was well above these background levels (~5-fold greater), which provides confidence as to the effect of genotype on expression phenotype. However, these levels preclude studies of agonist-promoted cAMP, since the majority of the transfected receptors are spare receptors.

In summary, the β_2 AR Arg16-containing haplotype is partitioned by the presence of 11, 12 or 13 C's within a poly-C-tract of the 3'UTR. The variable number of C's results in altered mRNA stability for one haplotype 11C leading to lower steady-state expression. This haplotype also displays wild-type agonist-promoted downregulation of expression, potentially leading to a low-response phenotype. Another variant (12C) has relatively stable mRNA and wild-type expression at baseline, and undergoes less agonist-promoted downregulation, potentially representing a high-response phenotype in regards to physiologic signaling. These phenotypes may represent important differences in the *in vivo* responses to β -agonists in the treatment of obstructive lung diseases.

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Fig. 1. Organization and relative location of the β_2 AR open-reading frame and 3'UTR polymorphisms. Numbering is based on the adenine of the initiator ATG designated as nucleotide 1.

Fig. 2.

Differential expression of β₂AR with variable poly-C 3'UTRs. BEAS-2B cells were transfected with constructs consisting of the β₂AR ORF with its 3'UTR containing 11, 12, or 13 C's in the positions shown in Table 1. In A), receptor protein expression was quantitated using ¹²⁵I-CYP radioligand binding, and the presence of 11 C's resulted in lower expression. Endogenous (mock-transfected) BEAS-2B cell membranes expressed 197 ± 13 fmol/mg of β₂AR. Results were normalized to the β₂Arg16-12C, which expressed the receptor at 1,507 ± 180 fmol/mg. In B), β₂AR mRNA was quantitated by real time RT-PCR; 11 C's was also found to have lower expression. Endogenous (mock-transfected) BEAS-2B β₂AR mRNA amounted to Ct values of ~28, while transfected β₂Arg16-12C had values of ~21, representing a ~100-fold increase in expression over background with transfection. Results are from 6 experiments. *, *P* < 0.01 vs. β₂Arg16-12C.

Fig. 3.

The poly-C tract polymorphisms alter $\beta_2\text{AR}$ mRNA degradation. BEAS-2B cells were transfected as described in Methods, and two days later treated with actinomycin D. Cells were harvested and mRNA prepared at the indicated times. The $\beta_2\text{Arg16-11C}$ curve differed from the $\beta_2\text{Arg16-12}$, and -13C curves ($P < 0.005$ by ANOVA). $\beta_2\text{Arg16-12C}$ and -13C had similar $t_{1/2}$ values, while $\beta_2\text{Arg16-11C}$ had an accelerated degradation rate with a lower $t_{1/2}$. The maximal extent of degradation over 6 hours was equivalent for the former two receptors, but greater for $\beta_2\text{Arg16-11C}$. See text for specific results for each haplotype. Shown are mean data from 6 experiments. *, $P < 0.05$ for maximal extent of degradation and $t_{1/2}$.

Fig. 4.

Effects of polymorphisms of the β_2 AR 3'UTR poly-C tract on agonist-promoted downregulation. Transfected BEAS-2B cells were exposed to vehicle (ascorbic acid) or vehicle with 10 μ M isoproterenol for 12 hrs at 37°C in serum-free media. Cell membranes were prepared and radioligand binding carried out as described in Methods. Results are from 8 experiments and are expressed as the percentage of downregulation imposed by agonist compared to vehicle treatment. *, $P < 0.02$ vs the other two haplotypes.

Table 1

Haplotype constructs of the β_2 AR with 3'UTR polymorphisms.*

Nucleotide →	46	79	252	491	523	1053	1239*	1266	1267	1268	1269	1270	1271	1272	1273	1274	1275	1276	1277	1278	1279
β_2 Arg16-11C	A	C	G	C	C	G	G	C	C	C				C	C	C	C	C	C	C	C
β_2 Arg16-12C	A	C	G	C	C	G	G	C	C	C			C	C	C	C	C	C	C	C	C
β_2 Arg16-13C	A	C	G	C	C	G	G	C	C	C		C	C	C	C	C	C	C	C	C	C

* Position 1269 is open to maintain numbering consistency, since 14-C's can rarely be found.