Variable-length poly-C tract polymorphisms of the β2-adrenergic receptor 3'-UTR alter expression and agonist regulation

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Submitted 17 July 2007; accepted in final form 7 November 2007

Panebra A, Schwarb MR, Swift SM, Weiss ST, Bleecker ER, Hawkins GA, Liggett SB. Variable-length poly-C tract polymorphisms of the β2-adrenergic receptor 3'-UTR alter expression and agonist regulation. Am J Physiol Lung Cell Mol Physiol 294:L190–L195, 2008. First published November 16, 2007; doi:10.1152/ajplung.00277.2007—β2-Adrenergic receptors (β2-AR) play 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 β2-AR agonists for prevention and acute treatment of bronchospasm in lung diseases such as asthma and chronic obstructive lung disease. The β2-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. On the basis of 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 position 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 physiological 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 β2-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 recently found (13) that Arg16 is partitioned into multiple haplotypes due to polymorphisms of a poly-C repeat region of the β2-AR 3'-untranslated region (UTR). Indeed, Caucasians with the Arg16 genotype represent three different haplotypes defined by the 3'-UTR 11, 12, or 13 Cs, with haplotype frequencies within the Arg16 group ranging from ~14% to 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 β2-AR. Studies were carried out with transfections of the human airway epithelial cell line BEAS-2B. Constructs consisted of contiguous sequence representing the β2-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 Cs 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...
steadystate cell surface expression of the β2-AR protein. In addition, the extent of agonistpromoted downregulation is dependent on the number of Cs in this region. 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 single nucleotide polymorphisms within the gene in association studies involving β2-ARrelated 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 β2-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 β2-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, 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 Cs) to 1279 (Table 1). Site-directed mutagenesis was utilized to alter the number of Cs within this region, resulting in a tract of 11, 12, or 13 Cs as is observed in the human population within the Arg16 haplotype (also referred to as haplotype 4; Ref. 7). The final expression constructs consisted of the β2-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 in Ham’s F-12 medium with 10% fetal calf serum as previously described (21). Transfections with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) were carried out as previously described (26). Briefly, ~10⁴ cells in monolayers in a T75 flask were treated with 5.0 μl of Lipofectamine reagent in 8 ml of serumfree medium for 6 h. Subsequently, 8 ml of additional medium with serum to provide a concentration of 10% was added, and the cells were harvested 12 h later.

Radioiodination. BEAS-2B cells endogenously express β-AR, which are ~95% of the β2-AR subtype (1), and the transfected receptors are also β2-AR, overexpressed by approximately five-to sevenfold over the endogenous background. Thus receptor expression, as quantitated with the high-affinity β-AR radioligand [125I]-cyanopindolol (125I-CYP, Perkin-Elmer, Waltham, MA), was considered all of the β2-AR subtype, given the <1% β1-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 g for 15 min, and the pellet was resuspended in 75 mM Tris, 12 mM MgCl₂, and 2 mM EDTA, pH 7.40. Radioligand binding with [125I]-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 h. Specific binding was normalized to protein and expressed as femtomoles per milligram of protein.

Quantitative RT-PCR. The remaining one-half of the flask of monolayered cells was treated with TRIZol reagent (Invitrogen) and frozen at −80°C, for batch extraction of RNA and DNase I 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 s and then cooled to 4°C. Real-time PCR was carried out with 1/50th of the reverse transcription reaction. The TaqMan probe and primer sets were from Applied Biosystems for the human β2-AR, which provide an amplicon of 66 bp representing nucleotides 500–565 of the β2-AR coding region. Similar reagents from Applied Biosystems were used to amplify GAPDH. Threshold cycle (Ct) values were quantitatively compared with β2Arg16-12C, or time 0 values as indicated, using the 2^-ΔΔCt method (20, 25).

Statistical analysis. Data are presented as means ± 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 β2Arg16-12C haplotype, so as to illustrate the relationship between haplotypes. The data were also fit by iterative techniques to a one-phase exponential decay to determine kinetic parameters (Prism, GraphPad, San Diego, CA).

RESULTS

Shown in Fig. 1 are the polymorphisms of the β2-AR 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 Cs within the poly-C region. These are designated as β2Arg16-11C, β2Arg16-12C, and β2Arg16-13C (the gap shown at position 1269 is present to maintain consistency in the numbering system, since very rarely have 14 Cs in the tract been noted; Ref. 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 β2-AR expression amounting to 197 ± 13 fmol/mg (n = 6). This latter quality implies that they have the

| Designation | 46 | 79 | 252 | 491 | 523 | 1053 | 1239 | 1266 | 1267 | 1268 | 1269* | 1270 | 1271 | 1272 | 1274 | 1275 | 1276 | 1277 | 1278 | 1279 |
|-------------|----|----|-----|-----|-----|------|------|------|------|------|-------|------|------|------|------|------|------|------|------|------|------|
| β2Arg16-11C| A   | C   | G   | C   | G   | G    | C    | C    | C    | C    | C      | C    | C    | C    | C    | C    | C    | C    | C    | C    | C    |
| β2Arg16-12C| A   | C   | G   | C   | G   | G    | G    | C    | C    | C    | C      | C    | C    | C    | C    | C    | C    | C    | C    | C    | C    |
| β2Arg16-13C| A   | C   | G   | C   | G   | G    | C    | C    | C    | C    | C      | C    | C    | C    | C    | C    | C    | C    | C    | C    | C    |

β2-AR, β2-adrenergic receptors; UTR, untranslated region. *Position 1269 is open to maintain numbering consistency, since 14 Cs can rarely be found.
necessary factors for regulation of receptor expression, thus potentially representing a better model cell compared with cells lacking endogenous \( \beta_2 \)-AR expression. The BEAS-2B cells transfected with the \( \beta_2 \text{Arg16-12C} \) haplotype expressed at an average level of 1,507 ± 180 fmol/mg (\( n = 6 \)), providing a sufficient level over background to compare the effects of the poly-C tract polymorphisms. The \( \beta_2 \)-AR haplotype expression constructs, representing contiguous sequence from the open reading frame to a distal 3′-UTR, were transfected into cells in parallel, and one-half of the culture plate was harvested for protein and the other for RNA 48 h after transfection (a time point when steady-state protein and mRNA levels are achieved; data not shown). \( \beta_2 \)-AR protein expression was determined by quantitative radioligand binding using \(^{125}\text{I-CYP} \). \( \beta_2 \)-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, where \( \beta_2 \text{Arg16-11C} \) has lower expression than the other two haplotypes (66 ± 2 Arg16-12C, being 55 ± 4% for \( \beta_2 \text{Arg16-12C} \) and \( \beta_2 \text{Arg16-11C} \) having lower expression than the other two haplotypes (66 ± 10%). Given the agreement between \( \beta_2 \)-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 \( \beta_2 \text{Arg16-11C} \) was 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 h after transfection; \( \beta_2 \)-AR mRNA half-life (\( t_{1/2} \)) was derived by determining mRNA by quantitative RT-PCR at various time points up to 6 h after actinomycin D treatment. The maximal extent of mRNA loss over 6 h was greatest for \( \beta_2 \text{Arg16-11C} \), being 55 ± 4% compared with 39 ± 5% and 44 ± 3% for \( \beta_2 \text{Arg16-13C} \) and \( \beta_2 \text{Arg16-12C} \), respectively (Fig. 3; \( P < 0.05 \)). The \( t_{1/2} \) was lower for \( \beta_2 \text{Arg16-11C} \) (0.62 ± 0.06, \( P < 0.05 \)) compared with those for the 13 C and 12 C 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, with the steady-state expression phenotypes shown in Fig. 2, where \( \beta_2 \text{Arg16-11C} \) has lower \( \beta_2 \)-AR mRNA and protein expression compared with the other two receptors.

We next considered whether agonist-promoted downregulation of \( \beta_2 \)-AR was affected by the poly-C tract. \( \beta_2 \)-AR expression is regulated by long-term agonist exposure by alterations in mRNA transcription or degradation, translation, internalization of receptors, and degradation of \( \beta_2 \)-AR protein (9). Thus for these assays expression was quantitated with \(^{125}\text{I-CYP} \).
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 μM of the β-AR agonist isoproterenol for 12 h, are shown in Fig. 4. Both β2Arg16-11C and -13C underwent equivalent downregulation amounting to a decrease of 21 ± 5.2 and 26 ± 5.3%, respectively (P > 0.10). In contrast, β2Arg16-12C underwent less agonist-promoted downregulation (14 ± 5.4%, P < 0.05 vs. either of the other two haplotypes).

DISCUSSION

As introduced earlier, the Arg16 polymorphism of the β2-AR has been associated with various β-agonist phenotypes in asthmatic patients (14, 15, 22, 30, 33). However, discrepancies in these findings from other studies are readily appreciated (4, 11, 32). Of interest have 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 by considering other genes in the β2-AR signaling pathway (29). One justification for the use of the polymorphism at position 16 within the β2-AR coding region has been that it defines a major haplotype, if one considers the 5′-flanking and coding polymorphisms. This haplotype, as described 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 Cs 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≈14%, 12C≈41%, and 13C≈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 β2Arg16-11C was decreased by 25% (protein expression) and 33% (mRNA expression) compared with β2Arg16-12C and -13C. When cells were treated with actinomycin to block mRNA transcription, β2Arg16-11C mRNA underwent substantially greater, and more rapid, degradation compared with the other two poly-C haplotypes. These data indicate that in the basal state β2Arg16-11C 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 with other Arg16-containing β2-AR haplotypes. It is interesting to note that a substantial variability in the in vivo β-agonist response has been reported in individuals with the homozygous Arg16-based 5′/coding haplotype [forced expiratory volume in 1 s (FEV1)%-predicted equal to 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 Cs in the 3′-UTR.

We also examined the effect of persistent β-agonist exposure on receptor downregulation between the variable C haplotypes. Agonist-promoted β2-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 β2Arg16-11C undergoes downregulation similar to the 13C haplotype. So, potentially, individuals with the β2Arg16-11C 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 with 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 β2-AR 3′-UTR lies close to (~20 bp downstream)
an AU-rich region, which is known to affect the rate of mRNA translation of β2-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 Cs) have an effect on the AU region, or the hairpin structure, is not predictable from current modeling techniques. In the cell-based system utilized here, we felt it was essential that the cells be of human origin, be a cell type of importance to asthma, and indeed express the β2-AR. The transfected β2-AR was well above these background levels which provides confidence as to the effect of genotype on expression phenotype. However, these levels preclude studies of agonist-promoted AMP, since the majority of the transfected receptors are spare receptors. In summary, the β2-AR Arg16-containing haplotype is partitioned by the presence of 11, 12, or 13 Cs within a poly-C tract of the 3'-UTR. The variable number of Cs 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 leading to a high-response phenotype in regard to asthma, and indeed express the β2-AR. The transfected β2-AR was well above these background levels which provides confidence as to the effect of genotype on expression phenotype. However, these levels preclude studies of agonist-promoted AMP, since the majority of the transfected receptors are spare receptors.

ACKNOWLEDGMENTS

We thank Esther Moses for manuscript preparation.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants HL-065899, HL-071609, and HL-045967.

REFERENCES


