Polymorphism of the β_2-Adrenergic Receptor Gene and Desensitization in Human Airway Smooth Muscle

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We examined the influence of two common polymorphic forms of the β_2-adrenergic receptor (β_2AR): the Gly16 and Glu27 alleles, on acute and long-term β_2AR desensitization in human airway smooth muscle (HASM) cells. In cells from 15 individuals, considered without respect to genotype, pretreatment with Isoproterenol (ISO) at 10^{-7} M for 1 h or 24 h caused approximately 25% and 64% decreases in the ability of subsequent ISO (10^{-6} M) stimulation to reduce HASM cell stiffness as measured by magnetic twisting cytometry. Similar results were obtained with ISO-induced cyclic adenosine monophosphate (cAMP) as the outcome indicator. Data were then stratified *post hoc* by genotype. Cells containing at least one Glu27 allele (equivalent to presence of the Gly16Glu27 haplotype) showed significantly greater acute desensitization than did cells with no Glu27 allele, whether ISO-induced cell stiffness (34% versus 19%, $p < 0.03$) or cAMP formation (58% versus 11%, $p < 0.02$) was measured. Likewise, cells with any Glu27 allele showed greater long-term desensitization of cell stiffness and cAMP formation responses than did cells without the Glu27 allele. The distribution of genotypes limited direct conclusions about the influence of the Gly16 allele. However, presence of the Gly16Gln27 haplotype was associated with less acute and long-term desensitization of ISO-induced cAMP formation than was seen in cells without the Gly16Gln27 haplotype (14% versus 47%, $p < 0.09$ for short-term desensitization; 32% versus 84%, $p < 0.01$ for long-term desensitization), suggesting that the influence of Glu27 is not through its association with Gly16. The Glu27 allele was in strong linkage disequilibrium with the Arg19 allele, a polymorphic form of the β_2AR upstream peptide of the 5′-leader cistron of the β_2AR, and this polymorphism in the β_2AR 5′-flanking region may explain the effects of the Glu27 allele. Cells with any Arg19 allele showed significantly greater acute and long-term desensitization of ISO-induced cAMP formation than did cells without the Arg19 allele (54% versus 2%, $p < 0.01$ for short-term desensitization; 73% versus 35%, $p < 0.05$ for long-term desensitization). Similar results were obtained for ISO-induced changes in cell stiffness. Thus, the presence of the Glu27 allele is associated with increased acute and long-term desensitization in HASM.

β-Agonists are the most widely used form of acute treatment for symptomatic asthma. Within the airway, binding of these agents to β_2-adrenergic receptors (β_2AR) in airway smooth muscle (ASM) results in bronchodilation. There are at least four missense single nucleotide polymorphisms in the coding region of the β_2AR (1). Two of them, an Arg→Gly mutation at position 16 and a Gln→Glu mutation at position 27, have been studied extensively because of their high prevalence in the population and the possibility that they may contribute to the pathogenesis of asthma (1–10). Although no polymorphism has been found to occur more commonly in asthmatic than in normal subjects, the Gly16 and Glu27 polymorphisms have been shown to influence the response to asthma treatment. The mechanistic basis for these associations remains to be established. One possibility is that endogenous catecholamines or inhaled β-agonists enhance downregulation when specific polymorphisms of the β_2AR are present. Green and colleagues reported that when Chinese hamster fibroblasts (which do not express β_2AR) are transfected with this receptor and exposed to the β-agonist isoproterenol (ISO) for 24 h, β_2AR expression decreases (11). In cells transfected with the Gly16 variant of the receptor, ISO causes a greater decrease in β_2AR number than in cells transfected with the wild-type receptor. In contrast, the Glu27 mutation is protective against ISO-induced decreases in β_2AR expression. Similar results were observed in cultured human airway smooth-muscle (HASM) cells (12).

The decrease in the β_2AR number caused by such long-term β-agonist exposure has been shown in other cell types to be the result of decreased transcription, decreased messenger RNA (mRNA) stability, and degradation of the β_2AR protein (13–16). Acute desensitization, in contrast, results from phosphorylation of the receptor and subsequent uncoupling of the receptor from G_s (17, 18). Because long-term and acute desensitization involve distinct mechanisms, the influence of polymorphisms of the β_2AR on long-term desensitization does not predict their influence on acute desensitization.

The purpose of this study was to characterize the effects of the Gly16 and the Glu27 β_2AR alleles on acute desensitization of the receptor in HASM cells, and to compare these effects with the effects of the two alleles on long-term desensitization. To assess desensitization, we measured functional outcomes, consisting of ISO-induced changes in cyclic adenosine monophosphate (cAMP) formation and ISO-induced changes in cell stiffness as measured by magnetic twisting cytometry (MTC), rather than measuring β_2AR number. Surprisingly, results with HASM cells derived from 15 different donors indicated that cells from individuals who were either homozygous or heterozygous for Glu27 showed both greater short- and long-term desensitization than occurred in cells from individuals who were homozygous for Gln27, even though Green and colleagues had previously reported that Glu27 is protective against desensitization (11, 12).

The Glu27 allele has been found to be in strong linkage disequilibrium with an unusual polymorphism in the 5′ flanking region of the β_2AR gene (19). A short open reading frame in

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the 5′ leader cistron (5′LC) encodes a 19–amino-acid peptide, termed the β_2AR-upstream peptide (BUP), and this peptide has been shown to inhibit β_2AR translation (20). A single nucleotide mutation results in an Arg→Cys mutation at amino acid 19 of the BUP, and the presence of Arg19 as compared with Cys19 has been associated with decreased β_2AR expression in transfected COS-7 and in cultured HASM cells (19). It is possible that the influence of Glu27 on desensitization results from its linkage disequilibrium with the Arg19 allele.

METHODS

Cell Culture

Human tracheas were obtained from lung transplant donors in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings. Tracheal smooth-muscle cells were harvested from the tracheas as previously described (21–24). The cells were then grown in plastic flasks in Ham's F12 medium with 10% fetal calf serum (FCS) that was supplemented with penicillin (100 U/ml), streptomycin (0.1 mg/ml), NaOH (12 mM), amphotericin-B (2.5 μg/ml), $CaCl_2$ (1.6 mM), and L-glutamine (2 mM). Medium was replaced every 3 to 4 d, and cells were passaged with 0.25% trypsin and 1 mM ethylene diamine tetraacetic acid (EDTA) every 10 to 14 days. Cells were studied in Passages 4 to 7.

Experimental Protocol

In HASM cells from 15 different donors, we examined short-term and long-term desensitization of the β_2AR by measuring the effects of pretreatment with ISO (10^{-7} M) for 1 h or 24 h on subsequent responses to ISO (10^{-6} M). We found this concentration of ISO (10^{-7} M) to induce significant but not maximal desensitization, both acutely and on a long-term basis, allowing us to make comparisons among treatment groups. Cell stiffness measured by MTC (see the subsequent discussion), and cAMP formation, were used as outcome indicators. For each donor, cells were studied on from two to six experimental days. For each set of experiments, two flasks of confluent HASM cells from the same passage were serum-deprived and supplemented with insulin at 5.7 μg/ml and transferrin at 5 μg/ml at 24 to 36 h before use, since these conditions maximize expression of smooth-muscle–specific contractile proteins (21, 25–27). At the same 24- to 36-h time point, both flasks were also treated with indomethacin (10^{-6} M). We used indomethacin to suppress differences in endogenous prostaglandin release among donors, since this variation might influence responses to ISO through heterologous desensitization (23). Approximately 3 h later, one flask was treated with ISO (10^{-7} M) and the other flask was not treated (control). On the morning of the experimental day (20 h after ISO treatment), cells were passaged and plated at: (*1*) 20,000 cells/well on collagen I (500 ng/cm^2)-coated bacteriologic plastic dishes (96-well Removawells; Immulon 2; Dynatech Laboratories, Chantilly, VA) for use in the MTC experiments; or (*2*) at 100,000 cells/well in 24-well plates for cAMP measurements. Indomethacin and ISO were added to appropriate wells. In approximately half of the untreated wells, ISO (10^{-7} M) was added 1 h before cell-stiffness measurements or before the cAMP assay, for the determination of short-term desensitization.

Cell-stiffness measurements with MTC were made from 2 to 10 h after plating, on an alternating basis among wells of the three study cell groups (untreated, treated with ISO for 24 h, treated with ISO for 1 h). On each experimental day, two or three wells of HASM cells from a single individual were studied in each treatment group. ISO was removed when magnetic beads were added 20 min before the cell-stiffness measurements. For cell-stiffness measurements, responses to ISO and dibutyryl-cAMP (db-cAMP) were obtained as follows: first, from two to four measurements of cell stiffness were made under baseline conditions. Beginning at 1 min after the addition of ISO (10^{-6} M), from two to four measurements of cell stiffness were again obtained. This concentration of ISO has been shown in other experiments to generate maximal responses to ISO. db-cAMP, at a concentration known to produce maximal effects (10^{-3} M), was added to the same wells, and from two to four measurements of stiffness were again made.

For cAMP measurements, from four to eight wells of HASM cells were plated for each treatment group. Cells were allowed to readhere for 4 h at 37° C, at which time the medium was replaced with 0.5 ml of phosphate-buffered saline containing 0.1 mM 3-isobutyl-1-methylxanthine and 300 μM ascorbic acid. ISO was not readded, and 30 min later, the cells either were treated with ISO (10^{-6} M) or were left untreated for measurement of basal cAMP formation. The cell supernatant containing cAMP was collected 10 min later, and cAMP was assayed with a Rainen [^{125}I]cAMP radioimmunoassay kit (New England Nuclear, Boston, MA) as previously described (22–24).

MTC

MTC was used to measure cytoskeletal mechanics of HASM cells, as previously described (22, 23, 28, 29). In this technique, ferromagnetic beads of 4.5-μm diameter and coated with a synthetic Arg-Gly-Asp (RGD)-containing peptide are added to wells containing HASM cells. The beads bind to cells through integrin receptors on the cell surface that recognize the RGD sequence. The wells containing the cells are placed within the magnetometer, where the beads are then magnetized with a brief 1,000-G pulse so that their magnetic moments are aligned in one direction, parallel to the surface on which the cells are plated. Subsequent application of a much smaller external magnetic field, orthogonal to the first field, produces a magnetic torque (or twisting force), in this case 80 dynes/cm^2, causing the beads bearing the cells to rotate as would a compass needle. Bead rotation is opposed, however, by reaction forces that develop within the cytoskeleton to which the beads are bound through the integrin receptors. MTC measures the resulting angular rotation (strain) of the magnetic bead in relation to the applied twisting stress, and the ratio of applied stress to strain is defined as the cell stiffness. Thus, the cell-stiffness measurements made by MTC reflect the resistance to shape distortion of the cytoskeleton, including that produced by actin and myosin. Consequently, stiffness increases with the application of contractile agonists and decreases after the addition of dilating agonists (28). However, although stiffness measurements made by MTC most likely reflect the effects of contractile and dilator agonists on actomyosin interactions, stiffness is likely to be a surrogate marker rather than a direct measure of the contraction and shortening of smooth muscle.

β_2AR Genotyping

DNA was isolated from HASM cells from each of the 15 donors through standard methods.

The region containing the β_2AR polymorphism at position 27 was amplified with the forward primer 5′–AACGGCAGCGCCTTCTTGCTG–3′ and the reverse primer 5′–AAAGGGCACCACTGCCAG–3′. Each PCR reaction contained 100 ng of DNA, PCR buffer with 1.5 mM $MgCl_2$ (Promega, Madison, WI), 200 μM deoxynucleotide triphosphates, and 10 pmol of each primer in a total volume of 25 μl of reaction mixture. Conditions for polymerase chain reaction (PCR) were 94° C for 6 min, followed by 35 cycles of 30 s at 94° C, 30 s at 62° C, and 30 s at 72° C, with a final extension time of 5 min at 72° C.

Restriction fragment length polymorphism (RFLP) was produced in 15 μl of PCR reaction mixture digested with 2 U of *Bbv* I and Buffer 2 (New England Biolabs, Boston, MA), according to the manufacturer's recommendations for 2 h at 37° C. The digest products were resolved by electrophoresis on a 2% agarose gel and stained with ethidium bromide. *Bbv* I digests only the Gln27 allele, to produce 180-bp and 51-bp fragments, whereas the uncut Glu27 allele gives a 231-bp fragment.

Genotyping at the β_2AR polymorphism at position 16 was done according to the method described by Martinez and coworkers (6). The β_2AR-16–containing region was amplified with the primers 5′–GCCTTCTTGCTGGCACCCCAT–3′ and 5′–CAGACGCTCGAACTTGGCCATG–3′. The underlined bases were modified from the reported sequence to create an *Nco* I site. The reverse primer contains an *Nco* I site and yields products from both alleles. The PCR product of the Gly16 allele contains the *Nco* I site in the 5′-primer. The PCR reaction conditions used for the β_2AR-16 genotyping study were similar to the conditions described for the β_2AR-27 study.

RFLP was produced in 10 μl of the PCR product digested with 2 U of *Nco*I and Buffer 4 (New England Biolabs) for 2 h at 37° C. The digest products were resolved by electrophoresis on a 4% NuSieve

(BioWhittaker Molecular Applications, Rockland, ME) agarose gel and stained with ethidium bromide. The uncut PCR product was 167 bp in length. *Nco*I digests the PCR product to produce a 145-bp fragment from the Arg16 allele and a 127-bp fragment from the Gly16 allele.

The accuracy of both genotyping methods was established by direct sequencing of PCR products amplified over the region of the receptor containing both polymorphic sites.

Haplotype assignments for the combination of amino acids 16 and 27 were made if homozygosity was present at either allele. For individuals heterozygous at both alleles, the region of the receptor containing both polymorphic sites was amplified by using the primers 5′–AACGGCAGCGCCTTCTTGCTG–3′ and 5′–CAGACGCTCGAACTTGGCCATG–3′, with PCR conditions identical to those used for amplification of the β_2AR-27 polymorphism. This PCR product was cloned with the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and direct sequencing was performed for haplotype determination.

Genotyping at the 5′LC polymorphism was done with a modification of the method described by McGraw and associates (19). This region was amplified with the primers 5′–GCTGAATGAGGCTTCCAGGC–3′ and 5′–CGCATGGCTTCTATTGGGTG–3′. Conditions for PCR were 94° C for 3 min, followed by 40 cycles of 45 s at 94° C, 45 s at 59° C, and 1 min at 72° C, with a final extension time of 7 min at 72° C.

RFLP was produced in 15 μl of PCR reaction mixture digested with 2 U of *Msp*A1I and Buffer 2 according to the manufacturer's recommendations, for 16 h at 37° C. The digest products were resolved by electrophoresis on a 4% NuSieve agarose gel and stained with ethidium bromide. *Msp*A1I digests only the Arg19 allele of the BUP to produce 103-bp and 60-bp fragments, whereas the uncut Cys19 allele was 167 bp in length.

Data Analysis and Statistics

Analysis of variance was used to determine statistical significance among the three treatment groups before the data were stratified by genotype. Linear mixed-effects models were used to assess whether, and the degree to which, genotype had a modifying effect on the mean shift in the outcome variable from baseline. The fixed-effects component of each model allowed the mean shift to be different for each group. The random-effects component mimicked the fixed-effects component according to the individual donor. The variance components for random effects were estimated by using restricted maximum likelihood via the function lme in the S-PLUS (MathWorks, Seattle, WA, 1996) software package. Values of p correspond to the significance of the mean modifying effect parameter obtained from that package. Data are presented as mean ± SE.

Reagents

Tissue culture reagents and drugs used in the study were obtained from Sigma (St. Louis, MO), with the exception of amphotericin-B and trypsin-EDTA solution, which were purchased from GIBCO (Grand Island, NY). Indomethacin was dissolved at 10^{-2} M in dimethylsulfoxide on each experimental day, and was then further diluted in medium before treating cells with it. ISO was dissolved at 10^{-1} M in distilled water on each experimental day, and because ISO is rapidly oxidized, dilutions of ISO in medium were made immediately before treating cells with ISO. db-cAMP was dissolved at 10^{-1} M in distilled water and frozen in aliquots until the day of use.

RESULTS

ISO Effects Not Stratified by Genotype

In untreated (control) HASM cells from 15 different donors, ISO (10^{-6} M) produced a response that was 76 ± 2% (mean ± SE) of the maximal decrease in cell stiffness (Figure 1). Maximal response was defined as the decrease in cell stiffness in response to db-cAMP (10^{-3} M), as has been previously described (22–24, 28), and did not differ among treatment groups. In cells pretreated with ISO (10^{-7} M) for 1 h and then washed for 20 min, subsequent ISO stimulation produced a significantly smaller response (57 ± 3% of maximal, $p < 0.0001$). In cells pretreated with ISO (10^{-7} M) for 24 h, the ISO response was even smaller (27 ± 3% of maximal, $p < 0.0001$). We then calculated the extent of desensitization from these values. For cells from all 15 donors, the degree of short-term (1 h) desensitization (25%) was less than half the degree of long-term (24 h) desensitization (64%).

Exposure to ISO for 1 h and for 24 h had effects on ISO-induced cAMP formation similar to those on cell stiffness. In untreated (control) cells, ISO (10^{-6} M) increased cAMP formation by 62.5 ± 5.8 pmol/10^6 cells above baseline values (22.1 ± 1.8 pmol/10^6 cells) (Figure 2), which was consistent with the approximately fourfold increase seen in untreated cells in previous experiments (22–24). Prior treatment with ISO (10^{-7} M) for 1 h resulted in significantly less of an increase in cAMP formation (42.6 ± 8.0 pmol/10^6 cells, $p <$

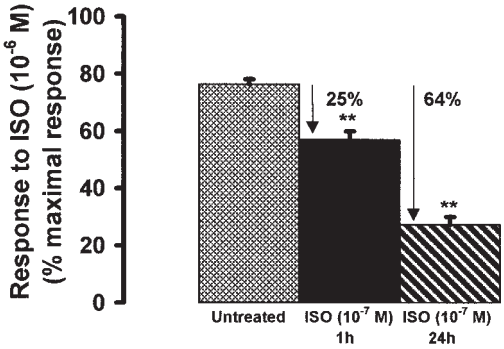

Figure 1. Effect of treatment with ISO (10^{-7} M for 1 h or 24 h) on changes in cell stiffness induced by subsequent ISO (10^{-6} M) stimulation. Response to ISO is expressed as the decrease in cell stiffness induced by ISO as a percent of the ultimate total decrease in cell stiffness after db-cAMP (10^{-3} M) stimulation. Data shown are mean ± SE obtained on 46 experimental days for 89 untreated wells, 73 wells treated with ISO for 1 h, and 74 wells treated with ISO for 24 h, in HASM cells obtained from 15 donors. **$p < 0.0001$ compared with untreated group. *Arrows* indicate degree of desensitization, which is defined as the difference between the ISO response of the untreated group and the ISO response of the 1-h or 24-h ISO pretreatment group divided by the ISO response of the untreated group.

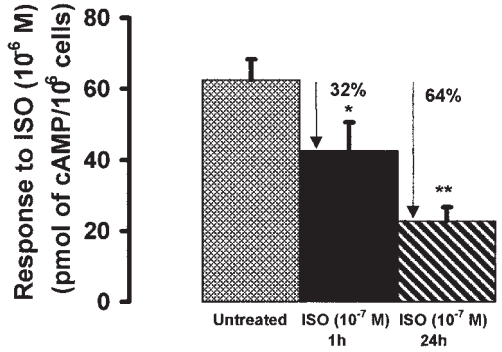

Figure 2. Effect of treatment with ISO (10^{-7} M for 1 h or 24 h) on ISO (10^{-6} M)-induced changes in cAMP formation. Response to ISO is expressed as the increase in cAMP formation in pmol of cAMP/10^6 cells induced by ISO above baseline cAMP formation. Results shown are mean ± SE of data obtained on 37 experimental days for HASM cells obtained from 15 donors in 77 and 81 untreated wells, in two sets of 52 wells treated with ISO for 1 h, and in 86 and 87 wells treated with ISO for 24 h. *$p < 0.001$, **$p < 0.0001$ compared with untreated cells. *Arrows* indicate degree of desensitization, which is defined as the difference between the ISO response of the untreated group and the ISO response of the 24- or 1-h ISO-pretreatment group divided by the ISO response of the untreated group.

TABLE 1

DISTRIBUTION OF β_2-ADRENERGIC RECEPTOR GENOTYPES

	Amino Acid 16			
	Arg/Arg	Arg/Gly	Gly/Gly	Total
Amino acid 27				
Gln/Gln	2	6	0	8
Gln/Glu	0	4	2	6
Glu/Glu	0	0	1	1
Total	2	10	3	

0.001), whereas prior treatment with ISO for 24 h resulted in even less of an increase in cAMP formation (22.7 ± 4.0 pmol/10^6 cells, $p < 0.0001$). Since cAMP formation at baseline did not differ among the treatment groups, desensitization was calculated from the ISO-induced increase in cAMP formation from baseline. The degree of acute (32%) and long-term (64%) desensitization in terms of ISO-induced cAMP formation was nearly identical to the degree of desensitization measured with cell-stiffness responses.

Data Stratified by Genotype

Table 1 shows the distribution of genotypes at amino acid positions 16 and 27 of the β_2AR for the HASM cells from the 15 donors in the study, and Table 2 shows the distribution of the haplotype combination for these genotypes. The distribution that we found was similar to what has been reported in the literature (1–10).

We stratified cell stiffness and cAMP responses to ISO by genotype and haplotype. Because there was no effect of genotype or haplotype on responses to ISO in untreated cells, values of ISO-induced cell-stiffness responses after 1 h or 24 h of ISO exposure were normalized, for each donor, as a percent of ISO-induced stiffness responses in untreated cells.

The presence of the Glu27 allele was associated with significantly greater acute desensitization (34 ± 6%) of ISO-induced changes in cell stiffness than was the absence of the Glu27 allele (19 ± 5%) (Figure 3, $p < 0.03$). Since the Arg16-Glu27 haplotype is rare (present in less than 1% of the population [6, 7, 9, 10, 30]) and was not present in our sample, presence (or absence) of the Glu27 allele in our data is equivalent to presence (or absence) of the Glu16Gln27 haplotype. Cells from donors with the Glu27 allele also showed significantly greater long-term desensitization (76 ± 6%) than did cells from donors without Glu27 (59 ± 5%) (Figure 3, $p < 0.02$). Similar results were obtained when we examined changes in ISO-induced cAMP formation (Figure 4). Cells from donors without the Glu27 allele exhibited virtually no acute desensitization (10 ± 12%), whereas the presence of Glu27 was associated with 58 ± 14% desensitization (Figure 4, $p < 0.02$). Although the difference was not statistically significant ($p = 0.18$), cells from individuals with the Glu27 allele also tended to exhibit greater long-term desensitization (70 ± 17%) than did cells from individuals without Glu27 (44 ± 13%) (Figure 4). The distribution of genotypes (only one donor was homozygous for Glu27) prevented a comparison between cells from donors homozygous for Glu27 and cells from donors heterozygous or homozygous for Gln27.

TABLE 2

DISTRIBUTION OF β_2-ADRENERGIC RECEPTOR HAPLOTYPES

Homozygous Arg16Gln27	2
Homozygous Gly16Glu27	1
Heterozygous Arg16Gln27, Gly16Gln27	6
Heterozygous Arg16Gln27, Gly16Glu27	4
Heterozygous Gly16Gln27, Gly16Glu27	2

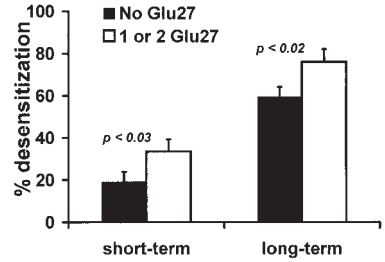

Figure 3. Effect of absence (no Glu27, *solid rectangles*) or presence (1 or 2 Glu27, *open rectangles*) of the Glu27 allele of the β_2AR on short-term (1 h) and long-term (24 h) desensitization to ISO-induced changes in cell stiffness in HASM cells. Results are expressed as % desensitization, and are the mean ± SE of ISO (10^{-6} M)-induced cell-stiffness responses in cells pretreated with ISO (10^{-7} M, 1 h or 24 h) as a percent of ISO-induced stiffness in untreated cells. Data were obtained in cells from eight donors with no Glu27 alleles and from seven donors with one or two Glu27 alleles.

We also examined the importance of the Gly16 polymorphism. The presence of Gly16 appeared to be associated with greater acute desensitization when cell stiffness was the outcome indicator, although the results were not statistically significant (Figure 5, $p < 0.07$). The presence of Gly16 did not appear to influence long-term desensitization of ISO-induced cell stiffness (Figure 5) or of ISO-induced cAMP formation (data not shown). The distribution of genotypes may have limited our ability to detect an effect of the Gly16 allele, since only two of the 15 HASM cell donors were homozygous for the Arg16 allele.

Since the presence of Glu27 was always associated with the presence of Gly16 in our population sample, we wanted to determine whether the effects of Glu27 resulted from its being in linkage disequilibrium with Gly16. To answer this question, given the distribution of haplotypes among our donors, we stratified our data, comparing results in the presence and absence of the Gly16Gln27 haplotype. Presence of the Gly16-Gln27 haplotype was associated with significantly less long-term desensitization of ISO-induced cAMP formation (32 ± 14%) than was absence of the Gly16Gln27 haplotype (82 ± 12%, $p < 0.01$) (Figure 6). Cells from donors with the Gly16Gln27

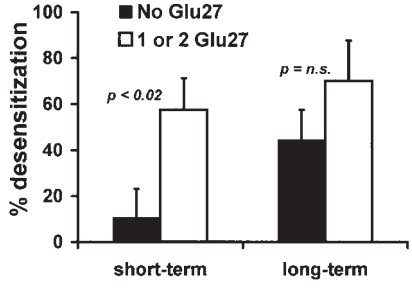

Figure 4. Effect of absence (no Glu27, *solid rectangles*) or presence (1 or 2 Glu27, *open rectangles*) of the Glu27 allele of the β_2AR on short-term (1 h) and long-term (24 h) desensitization to ISO-induced changes in cAMP formation in HASM cells. Results are expressed as % desensitization, and are the mean ± SE of ISO (10^{-6} M)-induced cAMP formation in cells pretreated with ISO (10^{-7} M, 1 h or 24 h) as a percent of ISO-induced cAMP formation in untreated cells. Data were obtained in cells from six to eight donors with no Glu27 alleles and from seven donors with one or two Glu27 alleles.

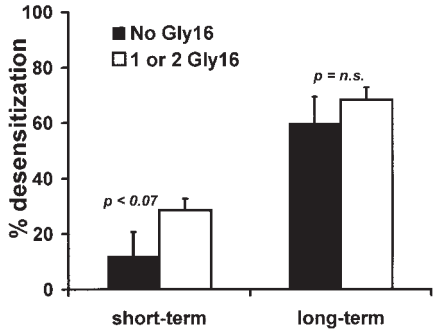

Figure 5. Effect of absence (no Gly16, *solid rectangles*) or presence (1 or 2 Gly16, *open rectangles*) of the Gly16 allele of the β_2AR on short-term (1 h) and long-term (24 h) desensitization to ISO-induced changes in cell stiffness in HASM cells. Results are expressed as % desensitization, and are the mean ± SE of ISO (10^{-6} M)-induced cell-stiffness responses in cells pretreated with ISO (10^{-7} M, 1 h or 24 h) as a percent of ISO-induced stiffness in untreated cells. Data were obtained in cells from two donors with no Gly16 alleles and from 13 donors with one or two Gly16 alleles.

haplotype also tended to exhibit less acute desensitization (47 ± 13%) than did cells from donors without the Gly16-Gln27 haplotype (82 ± 12%, $p < 0.09$). Taken together, these results suggest that the influence of Glu27 did not occur through its association with Gly16.

We also wanted to determine whether the effects of the Glu27 allele resulted from its being in linkage disequilibrium with the Arg19 allele of the BUP in the 5′ LC of the β_2AR, since the Arg19 polymorphism has been associated with decreased β_2AR expression in transfected COS-7 and in cultured HASM cells (19). The Arg19 polymorphism of the BUP has previously been shown to be in strong linkage disequilibrium with Glu27, whereas the Cys19 polymorphism has been shown to be in strong linkage disequilibrium with Arg16 (19). Our results (Table 3) were consistent with these previous observations. We then stratified our data according to the presence or absence of the Arg19 allele. Presence of the Arg19 allele was associated with significantly greater acute desensitization (54 ± 12%) of ISO-induced cAMP formation than was its absence (2 ± 14%) (Figure 7, $p < 0.01$). Cells from donors with the Arg19 allele also showed significantly greater long-term desensitization (73 ± 12%) than did cells from donors without the Arg19

TABLE 3

DISTRIBUTION OF THE 5′ LEADER CISTRON AND CODING BLOCK POLYMORPHISMS OF THE β_2-ADRENERGIC RECEPTOR

	Arg19	Het19	Cys19
Arg16	0	0	2
Het16	0	5	5
Gly16	1	2	0
Gln27	0	1	7
Het27	0	6	0
Glu27	1	0	0

allele (35 ± 16%, $p < 0.01$; Figure 7). Similar results were obtained for ISO-induced changes in cell stiffness (data not shown). Presence of the Arg19 allele was associated with greater acute ($p < 0.01$) and long-term ($p < 0.06$) desensitization.

DISCUSSION

Prior treatment of HASM cells with the β-agonist ISO for 1 h or 24 h decreased the cells' response to subsequent ISO stimulation, as assessed either with cell stiffness (Figure 1) or cAMP formation (Figure 2). The genotypes of the β_2AR expressed in the HASM cells from the 15 study donors were then determined *post hoc.* The presence of Glu27 (equivalent to the Gly16Glu27 haplotype) was associated with significantly greater acute desensitization, whether ISO-induced cell-stiffness responses (Figure 3) or ISO-induced cAMP formation (Figure 4) was used as the outcome indicator. Presence of the Glu27 allele was also associated with significantly greater long-term desensitization of ISO-induced cell-stiffness responses (Figure 3), and a similar trend was observed for ISO-induced cAMP responses (Figure 4). The distribution of genotypes, with only two donors homozygous for the Arg16 allele (Table 1), limited direct conclusions about the influence of the Gly16 allele (Figure 5). However, presence of the Gly16Gln27 haplotype was associated with significantly less acute and long-term desensitization, whether ISO-induced changes in cAMP formation (Figure 6) or cell stiffness (data not shown) were measured. We also confirmed that the Arg19 allele of the BUP in the 5′LC of the β_2AR is in strong linkage disequilibrium with the Glu27 allele (Table 3), and we showed that it is also significantly associated with increased desensitization to ISO-induced

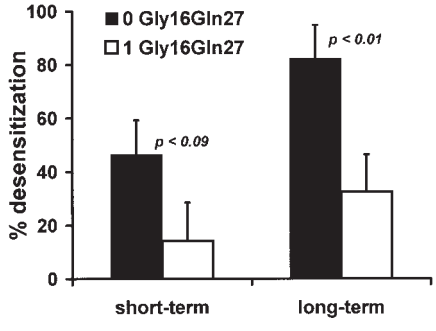

Figure 6. Effect of absence (no Gly16Gln27, *solid rectangles*) or presence (1 Gly16Gln27, *open rectangles*) of the Gly16Gln27 haplotype of the β_2AR on short-term (1 h) and long-term (24 h) desensitization to ISO-induced changes in cAMP formation in HASM cells. Results are expressed as % desensitization, and are the mean ± SE of ISO (10^{-6} M)-induced cAMP formation in cells pretreated with ISO (10^{-7} M, 1 h or 24 h) as a percent of ISO-induced cAMP formation in untreated cells. Data were obtained in cells from seven donors with no Gly16Gln27 and from six to eight donors with one Gly16Gln27.

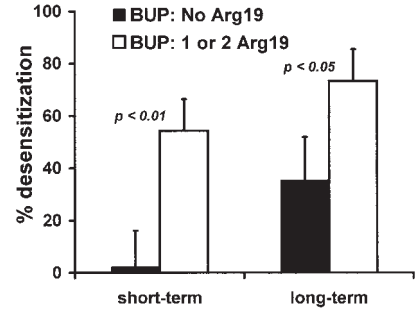

Figure 7. Effect of absence (no Arg19, *solid rectangles*) or presence (1 or 2 Arg19, *open rectangles*) of the Arg19 allele of the BUP in the 5′LC region of the β_2AR on short-term (1 h) and long-term (24 h) desensitization to ISO-induced changes in cAMP formation in HASM cells. Results are expressed as % desensitization, and are the mean ± SE of ISO (10^{-6} M)-induced cAMP formation in cells pretreated with ISO (10^{-7} M, 1 h or 24 h) as a percent of ISO-induced cAMP formation in untreated cells. Data were obtained in cells from eight donors without the Arg19 allele and from five to seven donors with the Arg19 allele.

changes in cAMP formation (Figure 7) and cell stiffness (data not shown).

In our sample, presence of the Glu27 allele was always associated with presence of the Gly16 allele, with the result that the effects of the Glu27 allele could have come from its being in linkage disequilibrium with Gly16. Ideally, an assessment of the role of the Glu27 allele could be achieved with a haplotype comparison of cells from donors homozygous for the Gly16Glu27 haplotype with cells from donors homozygous for the Gly16Gln27 haplotype. Given our limited sample size and the distribution of haplotypes in our donors, such a comparison was not possible, since even with 15 donors we did not find a single one who was homozygous for the Gly16Glu27 haplotype. However, when we performed a haplotype analysis in which we assessed the influence of the presence or absence the Gly16Glu27 haplotype, we saw increased desensitization in the presence of even a single Gly16Glu27 allele (Figures 3 and 4). In contrast, the presence of even a single Gly16Gln27 haplotype was associated with decreased desensitization (Figure 6). These data suggest that the influence of Glu27 does not occur through its association with Gly16.

Current understanding of how polymorphic forms of the β_2AR influence desensitization is based on data from only two studies, in which only long-term exposure was studied and β_2AR expression was the outcome indicator (11, 12). Our results extend the observations in those studies in several significant ways. First, we measured both acute and long-term desensitization. Acute desensitization is known to occur within minutes of continuous agonist exposure, as the result of phosphorylation and decoupling of the β_2AR from G_s (18), whereas desensitization over the longer term is associated with a decrease in receptor number as a result of decreased mRNA expression and increased receptor degradation (14–16). On the basis of the distinct mechanisms involved in acute and long-term desensitization, we reasoned that the role of β_2AR polymorphisms in short-term and long-term desensitization might not necessarily be the same. Corroborating this, our results indicated that the Glu27 allele was associated with increased short-term desensitization. To our surprise, we also observed that Glu27 was associated with increased long-term desensitization, even though Green and colleagues had previously reported that Glu27 is protective against desensitization (11, 12). We are confident of the validity of our results in that we used two independent functional indicators to assess desensitization (cell stiffness and cAMP formation), and found the same trend with both methods. Further, we measured responses in HASM cells from 15 individuals.

Two explanations are possible for the dichotomy between our results and those of Green and colleagues (11, 12). First, with respect to Glu27, the genotypes of our cells and those of Green and colleagues (12) were not the same. Their conclusion, that homozygosity for Glu27 is protective against desensitization, was based on observations in HASM cells from a single individual whose β_2AR contained the Glu27 allele in linkage with the Arg16 allele, giving the rare Arg16Glu27 haplotype, which is present in only about 1% of the population (6, 7, 9, 10, 30). In Green and colleagues' previous study, with transfected Chinese hamster fibroblasts, homozygosity for the Arg16Glu27 haplotype was protective against desensitization of β_2AR density, but cells homozygous for the Gly16Glu27 haplotype showed desensitization similar to that seen in wild-type cells (homozygous for Arg16Gln27) (11). The Arg16Glu27 haplotype was not present in our 15 donors.

The other potential reason for our findings is that receptor density may not be the most relevant marker for assessing β_2AR desensitization. Previous studies have shown that the physiologic responsiveness of the βAR system is not associated with a parallel change in βAR density. Nishikawa and coworkers exposed guinea pig lung to a β-agonist for 7 d and measured a 46% decrease in β_2 receptor density in ASM and a 75% decrease in β_2AR mRNA expression in ASM, but only a 10% decrease in maximum relaxation response to a β-agonist (14). Other studies have confirmed these findings, suggesting the presence of a substantial spare βAR population (31, 32). Although Green and colleagues emphasized increased desensitization of ISO-induced β_2AR expression, their results are consistent with ours in that they also saw no effect of Gly16 on long-term desensitization when ISO-induced cAMP accumulation, rather than β_2AR expression, was the outcome indicator (12).

We do not know the mechanism by which the presence of Glu27 increases the degree of acute and long-term desensitization in HASM cells. One possibility is that the coding block polymorphism has no primary effect but is in linkage disequilibrium with a polymorphism in the 5′-flanking region of the β_2AR that is important for desensitization. The Glu27 polymorphism has been shown to be in strong linkage disequilibrium with a Cys→Arg polymorphism in the BUP of the 5′LC of the human β_2AR gene (19). This Arg19 5′LC polymorphism has been shown to result in decreased β_2AR expression both in transfected COS-7 cells and in HASM cells. Therefore, the influence of Glu27 may be the result of decreased β_2AR expression at baseline, resulting in a cell that is more easily desensitized. In this study, we also demonstrated strong linkage disequilibrium between presence of the Arg19 polymorphism and presence of the Glu27 polymorphism (Table 3). As indicated, one donor homozygous for the Gln27 allele was heterozygous for Arg19Cys19, which accounts for the differences in desensitization reflected by cAMP formation seen in Figures 4 and 7. If anything, presence of the Arg19 polymorphism of the BUP of the 5′LC region was associated with at least as much long-term and acute desensitization (Figure 7) as was seen in the presence of the Glu27 allele (Figure 4), suggesting that the effects of Glu27 may result from its being in linkage disequilibrium with the Arg19 allele.

However, the influence of Glu27 on desensitization might also be the result of direct effects on the receptor, rather than of effects on its level of expression. In their study in transfected Chinese hamster fibroblasts, Green and colleagues detected no difference in the sequestration of receptors or in the distribution of cell surface versus internalized β_2AR receptors in the basal state among the four combinations of β_2AR polymorphisms at amino acids 16 and 27 (11). However, they demonstrated that β_2AR homozygous for the Glu27 allele had altered electrophoretic mobility as compared with wild-type β_2AR, suggesting that β_2AR with the Glu27 allele do not reach the mature wild-type conformation. Mutated β_2AR lacking amino acids 21 to 30 have been shown to be poorly processed by the cell to a mature protein (33). Thus, the addition of a negatively charged amino acid (Glu27) in the region from amino acids 21 to 30 may alter the ability of the receptor to reach the cell surface, leading to increased degradation of receptors after their initial synthesis or after internalization. In addition, McGraw and associates reported that long-term desensitization was attenuated with β_2AR lacking phosphorylation sites for β-adrenergic receptor kinase (βARK), one of the G-protein–related kinases known to be important in acute desensitization (34). Perhaps the presence of a negatively charged Glu27 induces a conformational change in the receptor, increasing either the ability of βARK to phosphorylate the receptor or of the receptor to remain phosphorylated, resulting in increased desensitization.

To our knowledge, only one clinical study has been reported in which β-agonist desensitization in human subjects was measured *in vivo.* That study demonstrated greater bronchodilator desensitization in the presence of Glu27 when forced expiratory flow from 25% to 75% of FVC was used as the clinical outcome indicator, which is in accord with our data (4). However, the *in vivo* study also found that Gly16 was associated with greater desensitization when FEV_1 was used as the outcome indicator. Since six of the 10 individuals in the *in vivo* study who were homozygous for Gly16 were also homozygous for Glu27, Glu27 was a prominent aspect of the Gly16 effect. Other clinical studies have sought an association between genetic polymorphisms of β_2AR and a variety of features that relate to asthma, including its prevalence, markers of asthma severity, and persistent bronchial hyperreactivity (1–3, 5–10), but the way in which asthma and airway reactivity are linked to β_2AR downregulation is not known.

The distribution of β_2AR genotypes and haplotypes in our study is fairly representative of that determined in larger population studies (1, 2, 4, 6–8, 30). Although only two donors were homozygous for Arg16, and this distribution limited direct comparisons with respect to the role of the Gly16 allele, the prevalence of Arg16 in the general population is virtually the same as in our population, at 13%. Another limitation was that our sample included no donors homozygous for the Gly16Gln27 haplotype. Not only would this haplotype be helpful in evaluating the role of the Gly16 allele, but also, two recent studies have suggested that presence of this haplotype is more prevalent in persons with moderate asthma (9) and is associated with measured bronchial hyperresponsiveness (10).

One caveat of our *in vitro* study is that ASM cells, after a few passages, may convert to a more synthetic phenotype, with the result that the influence of genotype on desensitization may not necessarily be the same in cultured HASM cells as it is *in situ.* We have previously demonstrated that nearly all cultured HASM cells (98 ± 2%) express smooth-muscle–specific α-actin, as determined immunochemically, and that both confluence and serum-free conditions increase actin expression (21). Although we do not know the extent to which β_2AR expression is altered in culture, the cAMP responses observed in cultured HASM cells are quantitatively similar to those observed in ASM preparations obtained *ex vivo* (25).

In conclusion, we have provided *in vitro* evidence that presence of the Glu27 allele of the β_2AR is associated with increased acute and long-term desensitization in HASM cells. This effect may result from linkage disequilibrium with a polymorphism in the 5′LC region of the β_2AR gene. Thus, the heterogeneity exhibited in response to asthma therapy may be influenced by specific genotypes of the β_2AR.

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