Differential Desensitization of Homozygous Haplotypes of the β_2 -Adrenergic Receptor in Lymphocytes

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Single-nucleotide polymorphisms of the β_2 -adrenergic receptor gene and its 5' promoter have been associated with differences in receptor function and desensitization. Linkage disequilibrium may account for inconsistencies in reported effects of isolated polymorphisms. Therefore, we have investigated the three most common homozygous haplotypes of the β_2 -adrenergic receptor (position 19 [Cys/Arg] of the 5' leader cistron and positions 16 [Arg/Gly] and 27 [Gln/Glu] of the receptor) for putative differences in agonist-induced desensitization. Lymphocytes of well defined nonasthmatic, nonallergic subjects homozygous for the haplotype CysGlyGln, ArgGlyGlu, or CysArgGln were isolated. Desensitization of (–)-isoproterenol-induced cyclic adenosine monophosphate (cAMP) accumulation and β_2 -adrenergic receptor sequestration and downregulation were measured in relation to β_2 -adrenergic receptor-mediated inhibition of IFN- γ and interleukin-5 production. We observed that lymphocytes of individuals bearing the CysGlyGln haplotype were more susceptible to desensitization of the β -agonist-induced cAMP response than those of individuals with the ArgGlyGlu or CysArgGln haplotype. The haplotype-dependent desensitization of β -agonist-induced cAMP response was not associated with haplotype-dependent β_2 -adrenergic receptor sequestration or downregulation. In addition, our data suggest reduced inhibition, in lymphocytes of subjects with the CysGlyGln haplotype, of interleukin-5 production induced by treatment with antibodies to the T-cell receptor-CD3 complex and to costimulatory molecule CD28 (α CD3/ α CD28). This is the first study demonstrating haplotype-related differences in agonist-induced β_2 -adrenergic receptor desensitization in primary human cells. This haplotype-related desensitization of the β_2 -adrenergic receptor in lymphocytes might have consequences regarding the regulation of helper T-cell type 2 inflammatory responses.

Keywords: 5' leader cistron; cAMP; cytokine production; sequestration and downregulation; single-nucleotide polymorphism

Functional β_2 -adrenergic receptors (β_2 ARs) are present on lymphocytes and β_2 AR-mediated antiinflammatory responses due to inhibition of proinflammatory cytokine production, chemotaxis, adhesion molecule expression, and proliferation have been described *in vitro* (1). Despite this, β_2 AR agonists have not been shown to be robust antiinflammatory agents in asthma treatment *in vivo*, as indicated by the poor effectiveness of these drugs to

resolve infiltration and activation of inflammatory cells, including eosinophils, lymphocytes, and macrophages, in the airways (2, 3). The apparent lack of these antiinflammatory effects could contribute to loss of disease control, loss of bronchoprotection, and rebound airway hyperresponsiveness after regular treatment with inhaled β_2 -agonists (4–6). One explanation for the poor antiinflammatory control could be a rapid development of agonist-induced β_2 AR desensitization in inflammatory cells, including lymphocytes (2, 7, 8). Because single-nucleotide polymorphisms (SNPs) of the β_2 AR gene and its 5' promoter have been associated with differences in receptor expression and desensitization susceptibility (9), they may have an impact on the antiinflammatory effects of β_2 AR agonists.

In vitro experiments in a recombinant system described increased and decreased agonist-induced β_2 AR downregulation for Arg16→Gly and Gln27→Glu, respectively (10). Agonist-induced β_2 AR desensitization in airway smooth muscle cells (11, 12) and mast cells (13) *in vitro*, and lymphocytes *ex vivo* (14, 15), have yielded conflicting results. Thus, Arg16Gly and Gln27Glu SNPs have been associated with either decreased or increased agonist-induced desensitization of β_2 AR-mediated cyclic adenosine monophosphate (cAMP) response (11, 12) or (onset of) downregulation (12–15). In addition, several more SNPs have been identified in the β_2 AR 5' promoter region, of which one is localized to the 5' leader cistron (5'LC), a signaling peptide involved in regulation of β_2 AR translation (16). In recombinant cells, 5'LC-Arg19→Cys (allelic frequency about 60%) leads to higher β_2 AR expression (16) and β_2 AR promoter-driven luciferase activity (17) when compared with wild type. A similar finding was obtained in airway smooth muscle cells (16).

In primary cells, both lack of statistical power and large linkage disequilibrium between β_2 AR SNPs may account for inconsistencies in reported effects of isolated SNPs. Therefore, haplotype analysis with sufficient numbers is needed to gain more powerful information regarding the functional significance of β_2 AR SNPs. In transiently transfected recombinant cells, clear differences were found in basal β_2 AR receptor expression between two common, naturally occurring haplotypes, bearing either CysArgGln or ArgGlyGlu at position 19 of the 5'LC and positions 16 and 27 of the receptor, respectively, which indeed correlated with lung function *in vivo* (18). By contrast, in peripheral lymphocytes of individuals with asthma, no haplotype differences were found in basal β_2 AR responsiveness and expression (19). So far, haplotype-dependent differences in agonist-induced β_2 AR desensitization and their possible functional consequences have not been studied in primary human cells.

In the present study, we investigated the hypothesis that haplotype-related differences in agonist-induced β_2 AR desensitization may have an impact on inflammatory mechanisms in asthma. This was accomplished by investigating agonist-induced β_2 AR desensitization and its effect on β_2 AR-mediated inhibition of IFN- γ and interleukin (IL)-5 production in peripheral lymphocytes from well-defined individuals homozygous for the naturally occurring haplotype CysGlyGln, ArgGlyGlu, or

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CysArgGln at position 19 of the 5'LC and positions 16 and 27 of the receptor. Part of this study has been presented in the form of an abstract (20).

METHODS

Subjects and Genotyping

Subjects were selected from a family study on the genetics of asthma (21). Subjects had to meet the following criteria: no allergy and no airway hyperresponsiveness, to exclude β_2 AR desensitization caused by allergen exposure (22); age between 18 and 45 years; and availability of genomic DNA. Genomic DNA of 125 selected subjects was genotyped prospectively for β_2 AR 5'LC and coding block SNPs as described (23). Haplotype analysis revealed 51 homozygous subjects (Table 1), 25 of whom consented to participate further in the study. The latter volunteers were subdivided into three groups based on their haplotype. Subject characteristics are summarized in Table 2. After 30 minutes of rest, 80 ml of blood was collected for isolation of lymphocytes. The study protocol was approved by the Medical Ethics Committee of the University of Groningen (Groningen, The Netherlands). Written informed consent was obtained from all participants. See the online supplement for additional detail on subject selection and genotyping.

Isolation of Lymphocytes

Lymphocytes were isolated from fresh blood by Ficoll-Hypaque density gradient centrifugation within 30 minutes of blood withdrawal (22) and resuspended in RPMI 1640 (Cambrex Bio Science, Verviers, Belgium) containing 10% fetal bovine serum, supplemented with penicillin (100 U/ml) and streptomycin (100 μ g/ml) (GIBCO-Life Technologies, Paisley, UK) at 5×10^6 cells/ml.

cAMP Accumulation

After pretreatment for 30 minutes and 2 hours with (–)-isoproterenol (1 μ M) or vehicle at 37°C, lymphocytes were immediately washed thoroughly at 4°C. Subsequently, (–)-isoproterenol (1 μ M)-induced cAMP accumulation was measured as described previously (24). See the online supplement for additional detail on the method for cAMP accumulation.

Receptor Sequestration and Downregulation

After pretreatment for 30 minutes, 2 hours, and 6 hours with (–)-isoproterenol (1 μ M) or vehicle at 37°C, lymphocytes were immediately washed at 4°C. β_2 AR sequestration and downregulation in intact lymphocytes were assessed by radioligand assays using (–)-[¹²⁵I]iodocyanopindolol in the absence or presence of either (–)-propranolol (1 μ M) or CGP 12177 (1 μ M) (25). See the online supplement for additional detail on the method for measuring sequestration and downregulation.

Inhibition of Cytokine Production

After pretreatment for 30 minutes and 2 hours with (–)-isoproterenol (1 μ M) or vehicle, lymphocytes were washed thoroughly at 4°C and

(–)-isoproterenol (1 μ M)-induced inhibition of α CD3/ α CD28 (antibodies to the T-cell receptor–CD3 complex and to costimulatory molecule CD28)-induced IFN- γ and IL-5 protein production for 24 hours was measured as described (26). Inhibition of α CD3/ α CD28-induced IFN- γ production by (–)-isoproterenol was completely blocked by 1 μ M timolol. See the online supplement for additional detail on the method for measuring inhibition of cytokine production.

Statistical Analysis

Data are expressed as median values (25th–75th percentiles), except for absolute cAMP values, which are presented as individual data and median values. Box plots represent median values and interquartiles, with bars representing minimum and maximum values. We were not able to assess reliable cAMP responses in three individuals. The same was true for radioligand binding in four subjects and IL-5 production in one subject. The missing data were unrelated to haplotype. Within-group differences were tested with the Wilcoxon signed rank test for paired observations. Mann-Whitney tests were used to evaluate differences between groups. Differences were considered significant when $p < 0.05$ (two-sided).

RESULTS

β_2 AR Haplotype Distribution

We confirmed large linkage disequilibrium between positions 16 and 27 of the β_2 AR and position 19 of the 5'LC (Table 1) (9). The extremely rare Arg16Glu27 haplotype was present in only one individual (<1%). This individual was heterozygous for the 5'LC and was therefore not included in the study. Moreover, subjects homozygous for 5'LC-Cys were all homozygous for Gln27. Three of the eight possible combinations of homozygous SNPs were present in our population.

(–)-Isoproterenol-induced Desensitization of cAMP Production

Pretreatment of lymphocytes with (–)-isoproterenol (1 μ M) for 30 minutes or 2 hours followed by extensive washout did not cause a significant change in basal cAMP levels (data not shown). However, a significant desensitization of (–)-isoproterenol-induced cAMP accumulation was observed at both time points (Figure 1A). As expected, the degree of agonist-specific desensitization after 2 hours was significantly higher than after 30 minutes (35.6 [28.2–44.6] vs. 58.9 [43.6–65.9]%; $p < 0.01$; Figure 1B). As we described earlier for lymphocytes of individuals with asthma (19), no haplotype differences were found in β_2 AR-induced cAMP responses, as observed in vehicle-treated cells after both 30 minutes and 2 hours of incubation (Figure 2A). Significant desensitization of (–)-isoproterenol-induced cAMP accumulation was observed for every β_2 AR haplotype both after 30 minutes and 2 hours of pretreatment with (–)-isoproterenol (Figure 2A). After 30 minutes of agonist pretreatment, median percentages of (–)-isoproterenol-induced desensitization of the CysGlyGln, ArgGlyGlu, and CysArgGln β_2 AR haplotypes were not significantly different (Figure 2B). In contrast, after 2 hours of pretreatment, the median percentage of agonist-induced desensitization in the CysGlyGln haplotype (69.5 [61.8–82.6]%) was significantly higher than in the ArgGlyGlu haplotype (57.5 [51.0–62.1]%; $p = 0.01$) and the CysArgGln haplotype (40.8 [3.3–59.9]%; $p = 0.03$; Figure 2B).

(–)-Isoproterenol-induced β_2 AR Sequestration and Downregulation

No significant differences were observed in basal β_2 AR density between all haplotypes at any of the time points studied (Table 3). Similarly, basal β_2 AR sequestration was not significantly different between haplotypes after 30 minutes and 2 hours of incubation with vehicle. Pretreatment with (–)-isoproterenol (1 μ M)

TABLE 1. β_2 -ADRENERGIC RECEPTOR HAPLOTYPE DISTRIBUTION

Haplotype			No.	Total (%)
5'LC19	aa 16	aa 27		
Cys/Arg	Arg/Gly	Gln/Glu	39	31.2
Arg/Arg	Gly/Gly	Glu/Glu	25	20.0
Cys/Cys	Arg/Arg	Gln/Gln	18	14.4
Cys/Cys	Arg/Gly	Gln/Gln	18	14.4
Cys/Arg	Gly/Gly	Gln/Glu	16	12.8
Cys/Cys	Gly/Gly	Gln/Gln	8	6.4
Cys/Arg	Arg/Arg	Glu/Glu	1	0.8

Definition of abbreviations: 5'LC19 = position 19 of the β_2 -adrenergic receptor 5' leader cistron; aa 16 = amino acid 16 of the β_2 -adrenergic receptor; aa 27 = amino acid 27 of the β_2 -adrenergic receptor.
n = 125 subjects.

TABLE 2. SUBJECT CHARACTERISTICS

Subject Characteristic	Haplotype			p Value
	CysGlyGln (n = 5)	ArgGlyGlu (n = 11)	CysArgGln (n = 9)	
Median age, yr (range)	37 (28–45)	33 (23–42)	24 (21–45)	0.68
Male/female	3/2	5/6	3/6	
Smoking status, non- or ex-smoker/current smoker	4/1	7/4	6/3	
Median FEV ₁ , % predicted (range)	99.5 (77.0–118.6)	92.9 (80.1–125.5)	93.7 (63.8–99.6)	0.54
IgE, IU/ml (range)	28 (2–35)	26 (10–66)	15 (2–28)	0.05
AHR, PC ₂₀ histamine	> 32 mg/ml	> 32 mg/ml	> 32 mg/ml	

Definition of abbreviations: AHR = airway hyperresponsiveness; PC₂₀ histamine = provocative concentration of histamine causing a 20% fall in FEV₁.

n = 25 subjects.

significantly increased β_2 AR sequestration at both time points (data not shown). In contrast to the observed haplotype differences in desensitization of cAMP responses (Figure 2B), agonist-induced β_2 AR sequestration was not different between the three haplotypes, both after 30 minutes and 2 hours of pretreatment (data not shown). A substantial increase in (–)-isoproterenol-induced downregulation became apparent only after 6 hours of

pretreatment (Figure 3A). As with sequestration, the extent of (–)-isoproterenol-induced downregulation was not haplotype dependent (Figure 3B).

(–)-Isoproterenol-induced Inhibition of IFN- γ and IL-5 Production

In accordance with previous findings in T cells (26), (–)-isoproterenol significantly inhibited α CD3/ α CD28-induced IFN- γ production (Figure 4A). Pretreatment of lymphocytes with (–)-isoproterenol for 30 minutes or 2 hours followed by extensive washout did not cause a significant change in absolute α CD3/ α CD28-induced IFN- γ as well as IL-5 production measured in the subsequent absence of β -agonist, when compared with vehicle-pretreated cells (data not shown). Interestingly, pretreatment of lymphocytes with (–)-isoproterenol (1 μ M) for both 30 minutes and 2 hours caused a significant decrease in the ability of the same agonist to inhibit α CD3/ α CD28-induced IFN- γ production (Figure 4A). Moreover, the desensitization of β_2 AR-mediated IFN- γ inhibition after 2 hours was significantly higher than after 30 minutes of pretreatment ($p = 0.001$), which was in full agreement with the agonist-induced desensitization of β_2 AR-mediated cAMP accumulation (Figure 1B). As published previously (27), (–)-isoproterenol also significantly inhibited IL-5 production (Figure 4B). Unlike β_2 AR-mediated IFN- γ inhibition, however, there were no significant effects of prior agonist treatment. As might be expected, no haplotype-related differences were observed in absolute α CD3/ α CD28-induced production of IFN- γ and IL-5 at any time point in the absence of (–)-isoproterenol (control; data not shown). In addition, no haplotype differences were observed in (–)-isoproterenol-induced inhibition of IFN- γ production in any of the pretreatment groups (Figure 5A). Remarkably, a small, but significant, reduction of (–)-isoproterenol-induced inhibition of IL-5 production was found in the CysGlyGln haplotype compared with ArgGlyGlu after 30 minutes and 2 hours of pretreatment with agonist and vehicle, respectively, whereas a trend was observed under the other conditions (Figure 5B). Moreover, after 2 hours of pretreatment with either vehicle or (–)-isoproterenol, lymphocytes of individuals bearing the CysGlyGln haplotype were not responsive for β_2 AR-mediated inhibition of IL-5 production at all (Figure 5B).

Figure 1. (–)-Isoproterenol-induced desensitization of β_2 -adrenergic receptor (β_2 AR)-mediated cyclic adenosine monophosphate (cAMP) accumulation in lymphocytes. (A) Effects of 30 minutes and 2 hours of pretreatment with vehicle (veh) or (–)-isoproterenol (iso) on β_2 AR-mediated cAMP accumulation induced by (–)-isoproterenol (1 μ M). (B) Percentage (–)-isoproterenol-induced desensitization of cAMP accumulation after 30 minutes and 2 hours of pretreatment. * $p < 0.01$; ** $p < 0.0001$. Data were obtained in lymphocytes from 22 subjects.

DISCUSSION

Despite inhibitory effects on inflammatory cells (1), β_2 AR agonists have not been shown to be potent antiinflammatory drugs in the treatment of asthma (2, 3). A likely explanation for this lack of control is agonist-induced desensitization (2). In the present study, we observed distinct differences in agonist-induced

Figure 2. (–)-Isoproterenol-induced desensitization of cAMP accumulation after 30 minutes and 2 hours of pretreatment, stratified for β_2 AR haplotype. (A) Effects of 30 minutes (top) and 2 hours (bottom) of pretreatment with vehicle (veh) or (–)-isoproterenol (iso) on β_2 AR-mediated cAMP accumulation induced by (–)-isoproterenol (1 μ M), stratified for β_2 AR haplotype. (B) Percentage of (–)-isoproterenol-induced desensitization of cAMP accumulation after 30 minutes (shaded boxes) and 2 hours (open boxes) of pretreatment, stratified for β_2 AR haplotype. Data were obtained in lymphocytes from five subjects with the CysGlyGln haplotype, 10 subjects with the ArgGlyGlu haplotype, and seven subjects with the CysArgGln β_2 AR haplotype. * $p < 0.05$ for differences between pretreatment conditions; # $p = 0.03$ and ## $p = 0.01$ for differences between haplotypes.

lymphocyte β_2 AR desensitization between three naturally occurring homozygous haplotypes of the β_2 AR gene and its 5' promoter, which may have impact on the regulation of helper T-cell type 2 (Th2) inflammatory responses.

Several polymorphisms of the β_2 AR and its 5' promoter have

TABLE 3. BASAL β_2 -ADRENERGIC RECEPTOR DENSITY IN VEHICLE-TREATED CELLS

Incubation Time	Haplotype		
	CysGlyGln (n = 5)	ArgGlyGlu (n = 9)	CysArgGln (n = 7)
30 min	2.54 (1.30–3.55)	2.56 (1.73–4.07)	2.62 (1.39–3.34)
2 h	1.37 (0.90–3.75)	2.15 (1.47–4.08)	2.23 (0.84–3.56)
6 h	2.92 (1.15–3.62)	2.19 (1.48–4.45)	2.03 (1.38–3.24)

Data are expressed as median (25th–75th percentiles) total specific [¹²⁵I]iodocyanopindolol binding in femtomoles per 10⁶ cells. Differences between haplotypes were nonsignificant at all time points.

been implicated in agonist-induced desensitization (9). In primary cells, linkage disequilibrium may account for conflicting results regarding functional effects of these polymorphisms when considered in isolation (11–15). Therefore, investigating naturally occurring β_2 AR haplotypes is elemental for assessing the functional significance of β_2 AR SNPs.

Because of their relatively easy accessibility, isolated lymphocytes are a major source of primary cells, in which haplotype-related differences in agonist-induced desensitization and its possible functional consequences can be studied prospectively in sufficient numbers within a clearly defined population. Prior treatment of lymphocytes with (–)-isoproterenol for 30 minutes and 2 hours caused a time-dependent reduction of (–)-isoproterenol-induced cAMP accumulation. After 2 hours of agonist treatment, the desensitization of β_2 AR-mediated cAMP production was significantly higher in lymphocytes from individuals bearing the CysGlyGln haplotype than in individuals with the ArgGlyGlu or CysArgGln haplotype. *In vitro* studies investigating functional effects of β_2 AR polymorphisms have focused mainly on long-term agonist-induced β_2 AR desensitization, that is, after 24 hours of pretreatment with a β -agonist (10–13). So far, only one study has investigated genotype-related differences in short-term β -agonist-induced desensitization, that is, after 1 hour of prior treatment (11). In the latter study, the presence of a Glu27 allele of the β_2 AR was associated with increased (–)-isoproterenol-induced desensitization of β_2 AR-mediated cAMP production and reduction of cell stiffness in airway smooth muscle cells. Of note, because of known linkage, the Glu27 allele was always present in combination with Gly16. Moreover, there were no individuals included who carried the homozygous Gly16Gln27 variant (11). Interestingly, we showed that the homozygous CysGlyGln haplotype was actually the one most susceptible to agonist-induced desensitization after 2 hours of agonist incubation. This might explain the difference between our results and those obtained in the study of Moore and coworkers (11) and stresses the importance of haplotype analysis in investigating functional consequences of β_2 AR polymorphisms.

We did not find any haplotype-related differences in basal β_2 AR density. This is in good agreement with previously published data on lymphocyte β_2 AR number with different polymorphisms at positions 16 and 27 of the receptor when considered in isolation (15, 28), and with different haplotypes at positions –367 and –47 (5'LC) of the 5' promoter and positions 16 and 27 of the receptor (19). By contrast, in recombinant COS-7 and HEK-293 cells, the 5'LC-Arg19→Cys polymorphism led to higher β_2 AR expression (16) and β_2 AR promoter-driven luciferase activity (17), respectively, when compared with wild type. However, the effects of 5'LC-Cys19 in the recombinant β_2 AR gene were studied in association with Gly16 and Glu27 (16), resulting in a haplotype that does not naturally occur. Alternatively, in transiently transfected HEK-293 cells the CysArgGln haplotype was associated with lower receptor protein and mRNA

Figure 3. (–)-Isoproterenol-induced β_2 AR downregulation. (A) (–)-Isoproterenol (1 μ M)-induced β_2 AR downregulation after 30 minutes, 2 hours, and 6 hours of treatment. * $p < 0.02$ compared with 30 minutes and 2 hours of pretreatment. Data were obtained in lymphocytes from 21 subjects. (B) β_2 AR downregulation after 6 hours of treatment with (–)-isoproterenol, stratified for β_2 AR haplotype: five subjects with the CysGlyGln haplotype, nine subjects with the ArgGlyGlu haplotype, and seven subjects with the CysArgGln β_2 AR haplotype.

expression when compared with ArgGlyGlu (18). Again, these observations emphasize the importance of investigating naturally occurring haplotypes in primary cells.

We observed clear (–)-isoproterenol-induced lymphocyte β_2 AR sequestration. So far, possible genotype-related differences in homologous β_2 AR sequestration have been studied only in recombinant CHW-1102 fibroblasts (10). Whereas in these cells, the Gly16Glu27 variant showed slightly less maximal sequestration than did the Arg16Glu27 and Gly16Gln27 variants, we did not find haplotype-related differences in both basal and (–)-isoproterenol-induced β_2 AR sequestration in human lymphocytes. In addition, no haplotype differences were observed in (–)-isoproterenol-induced downregulation at 6 hours of treatment. In the studies by Green and coworkers (10, 12), Gly16 polymorphism was associated with increased downregulation, whereas Glu27 protected against downregulation in CHW-1102 cells (10) as well as in airway smooth muscle cells (12). In lymphocytes, genotype-related differences in long-term agonist-induced downregulation have been studied only *ex vivo*, that is, after prolonged agonist treatment *in vivo*, and the results were rather divergent. Thus, in subjects with asthma, and homozygous for Gly16, there was a tendency toward increased downregulation after 1 week of treatment with inhaled formoterol (14). In healthy volunteers homozygous for Glu27, the onset of β_2 AR downregulation after 72 hours of treatment with oral terbutaline was delayed, whereas the final extent of β_2 AR downregulation, after 2 weeks of treatment, was genotype independent (15).

According to the observed differences between the CysGlyGln and CysArgGln haplotypes in our study, Gly16 seems to be responsible for the increased susceptibility to agonist-induced desensitization of β_2 AR-mediated cAMP production. However, when Gly16 was associated with 5'LC-Arg and Glu27, no significant differences were found between Arg16 and Gly16. Because the effect of position 16 was largely dependent on the combina-

Figure 4. (–)-Isoproterenol (1 μ M)-induced inhibition of α CD3/ α CD28-induced IFN- γ and interleukin (IL)-5 production. Effects of 30 minutes and 2 hours of pretreatment with vehicle (shaded boxes) or (–)-isoproterenol (1 μ M; open boxes) on (–)-isoproterenol-induced inhibition of IFN- γ (A) and IL-5 production (B). (–)-Isoproterenol significantly inhibited α CD3/ α CD28-induced IFN- γ and IL-5 production under all treatment conditions ($p < 0.005$). * $p = 0.005$ and ** $p < 0.0001$ compared with vehicle. Data for IL-5 were obtained in lymphocytes from 24 subjects. Data for IFN- γ were obtained from 25 subjects.

tion of SNPs at position 19 of the 5'LC and at position 27 of the receptor, our data point out the importance of considering haplotypes in investigating functional consequences of β_2 AR polymorphism.

Brief agonist exposure will lead to β_2 AR phosphorylation by β -adrenergic receptor kinases and cAMP-dependent protein kinase A (PKA). Phosphorylation by β -adrenergic receptor kinases and subsequent β -arrestin binding to the β_2 AR will lead to uncoupling of the receptor from $G\alpha_s$ and sequestration via clathrin-coated pits, which may precede recycling as well as downregulation of the β_2 AR (29). PKA-induced phosphorylation also leads to uncoupling, but without sequestration (30). Our results indicated that agonist-induced desensitization of cAMP production is haplotype dependent, but we did not find haplotype-related differences in agonist-induced sequestration. It is therefore tempting to speculate that, in lymphocytes, β_2 AR SNPs may have particular consequences for PKA-induced uncoupling. However, the role of PKA needs further confirmation, because agonist-induced downregulation (which could involve PKA activity by pretranslational changes in β_2 AR expression) was also haplotype independent.

A biochemical explanation for the observation that distinct polymorphisms within the extracellular amino terminal of β_2 AR contribute to the observed differences in agonist-induced desensitization is presently not at hand. Interestingly, however, both polymorphisms are located within (Arg16Gly) or in close proximity to (Gln27Glu) two tripeptide (N-X-S/T) consensus sites for N-linked glycosylation, which have been demonstrated to be involved both in cell surface expression of the β_2 AR (31) and in coupling of the receptor to $G\alpha_s$ (31, 32).

Figure 5. (–)-Isoproterenol (1 μ M)-induced inhibition of IFN- γ and IL-5 production after pretreatment with the same agonist concentration (open boxes) or vehicle (shaded boxes) for 30 minutes and 2 hours, stratified for β_2 AR haplotype. The numbers 1, 2, and 3 at the abscissa represent haplotypes CysGlyGln, ArgGlyGlu, and CysArgGln, respectively. (A) (–)-Isoproterenol significantly inhibited IFN- γ production in every haplotype under all treatment conditions ($p < 0.05$). No haplotype-related differences were found. Data were obtained in lymphocytes from five subjects with the CysGlyGln haplotype, 11 subjects with the

ArgGlyGlu haplotype, and nine subjects with the CysArgGln β_2 AR haplotype. (B) (–)-Isoproterenol significantly inhibited IL-5 production for every haplotype at the indicated time points ($p < 0.05$), except for those indicated by the following: + $p = 0.08$ and ++ $p = 0.5$. * $p < 0.05$ and § $0.05 < p \leq 0.1$ for differences between haplotypes. Data were obtained in lymphocytes from five subjects with the CysGlyGln β_2 AR haplotype, 11 subjects with the ArgGlyGlu β_2 AR haplotype, and eight subjects with the CysArgGln β_2 AR haplotype.

In accordance with previously published data (26, 27, 33), we found β_2 AR-mediated inhibition of IFN- γ and IL-5 production by α CD3/ α CD28-stimulated lymphocytes, which was relatively small for IL-5. The functional consequences of agonist-induced β_2 AR desensitization regarding inhibition of cytokine production have thus far not been addressed. Prior treatment with (–)-isoproterenol indeed caused desensitization of β_2 AR-mediated inhibition of α CD3/ α CD28-induced IFN- γ production. Moreover, the rate of attenuation of β -agonist-induced inhibition of IFN- γ production was strikingly similar to that observed for agonist-induced cAMP accumulation. In contrast, no significant desensitization was observed for the β_2 AR-mediated inhibition of IL-5 production. Both the relatively small inhibitory effect of (–)-isoproterenol on IL-5 production and the lack of desensitization of this effect might be related to the absence of a cAMP response element in the promoter sequence of the IL-5 gene (27, 33). By contrast, the expression of IFN- γ is under the control of a cAMP response element (34). An alternative explanation for the differential β_2 AR-mediated regulation of IFN- γ and IL-5 might be that inhibition of the IL-5 response was already attenuated as a consequence of the experimental procedure. Thus, to assess β_2 AR-mediated inhibition of cytokine production, cells were cultured in the presence of (–)-isoproterenol for 24 hours to obtain a measurable induction of α CD3/ α CD28-induced cytokine production. It might be envisaged that, because of differential regulating mechanisms, the long-term incubation itself caused a complete or almost complete desensitization of the β -agonist-induced inhibition of IL-5 production, whereas this was not observed for IFN- γ . In support of this latter explanation, the inhibition of IL-5 production tended to be reduced or even was completely absent in the CysGlyGln haplotype, both in vehicle- and agonist-treated cells. Of note, the observed haplotype differences regarding β_2 AR-mediated IL-5 inhibition are in agreement with the increased susceptibility of CysGlyGln to agonist-induced β_2 AR desensitization. Remarkably, despite the observation that agonist-induced reduction of β_2 AR-mediated IFN- γ inhibition was associated with agonist-induced desensitization of cAMP production and β_2 AR sequestration, no signifi-

cant haplotype-dependent differences were observed regarding the regulation of this cytokine.

Overall, our *in vitro* data suggest that individuals bearing the CysGlyGln haplotype are more prone to agonist-induced loss of β_2 AR responsiveness, possibly resulting in reduced control of Th2 cytokine production, which could contribute to the adverse effects of β_2 AR agonists (35). However, extrapolating *in vitro* data to the *in vivo* situation has thus far proved difficult. Whereas the Gly16 variant of the β_2 AR is associated with increased down-regulation *in vitro* (10, 12), the Arg16 variant appears to be associated with a reduced response to β_2 AR agonists (36) and a higher exacerbation rate (37) during treatment with albuterol *in vivo*. Moreover, individuals bearing the Arg16Gln27 haplotype showed significant (–)-isoproterenol-induced vascular desensitization of the hand vein, whereas individuals bearing Gly16Gln27 and Gly16Glu27 did not (38). The differences between *in vitro* and *in vivo* data could be explained by assuming that β_2 ARs of individuals bearing Gly16 are already desensitized by endogenous catecholamines (9). In this regard, proinflammatory cytokines in the asthmatic condition known to cause heterologous desensitization of the β_2 AR (39) might also be of importance.

Interestingly, two studies have found that the Gly16Gln27 haplotype was more prevalent in persons with moderate asthma than in persons with mild asthma (40) and was associated with bronchial hyperresponsiveness in a highly homogeneous sample of individuals (41).

In conclusion, this is the first study demonstrating haplotype-related differences in agonist-induced β_2 AR desensitization and its functional significance in primary human cells. In peripheral lymphocytes, we assessed the three most prevailing homozygous haplotypes of the β_2 AR at position 19 of the 5'LC and at positions 16 and 27 of the receptor. The CysGlyGln haplotype was more susceptible to agonist-induced desensitization of the β_2 -adrenergic cAMP response than were ArgGlyGlu and CysArgGln. This differential haplotype effect may have consequences regarding the regulation of Th2 inflammatory responses.

Conflict of Interest Statement: None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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