β_2-Adrenergic Receptor Genotype-Related Changes in cAMP Levels in Peripheral Blood Mononuclear Cells after Multiple-Dose Oral Procaterol

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Purpose. To evaluate the β_2-adrenergic receptor (β_2AR) genotype frequency in the Japanese population and the relationship between β_2AR genotype at amino acid position 16 (β_2AR-16) and desensitization to β_2-agonist *ex vivo*.

Methods. The β_2AR genotypes at amino acid positions 16, 27, and 164 of 92 healthy Japanese subjects were determined by polymerase chain reaction–restriction fragment-length polymorphism. The relationship between the β_2AR-16 genotype and the desensitization to β_2-agonist was examined in 10 male subjects *ex vivo*. Procaterol tablet (HCl salt, 50μg, Meptin®) was given orally for 5 days, and peripheral blood was obtained before and after 5 days of consecutive medications followed by the assessment of the intracellular cAMP levels in peripheral blood mononuclear cells after incubation with or without procaterol hydrochloride (0–1000 ng/mL).

Results. Allele frequency was Arg16:Gly16 = 46%:54%, Gln27:Glu27 = 92%:8%, and Thr164:Ile164 = 100%:0%, respectively. The cAMP levels were increased by incubation with procaterol hydrochloride, and the increase was suppressed after 5 days of consecutive medications. The suppression was more significant in the homozygote for Gly16 than the homozygote for Arg16.

Conclusions. The desensitization to β_2-agonist was associated more frequently with the mutation at β_2AR-16 (Gly16).

KEY WORDS: β_2-Adrenergic receptor; genotype; desensitization; procaterol; cAMP; peripheral blood mononuclear cells.

INTRODUCTION

β_2-Adrenergic receptor (β_2AR) agonists have been used widely as bronchodilators in asthma patients. Long-term and regular β_2-agonist therapy have been shown to produce desensitization and, consequently, excess use (1). The large interindividual variability in the desensitization is also an unresolved issue. Although the desensitization has been suggested to result from β_2-agonist-induced down-regulation and/or agonist-promoted uncoupling between the β_2AR and G_s-binding protein, no rational explanation has been proposed for the desensitization and its large interindividual variability. In the 1990s, gene polymorphisms of β_2AR were found, and β_2AR genotypes at amino acid position 16 (β_2AR-16; Arg → Gly), at position 27 (β_2AR-27; Gln → Glu), and at position 164 (β_2AR-164; Thr → Ile) were shown to be responsible for receptor functions after agonist exposure (2). *In vitro,* Gly16 has been shown to be associated with enhanced β_2-agonist-induced receptor down-regulation, whereas Glu27 seems to protect against this down-regulation (2,3). However, agonist binding and coupling to adenylate cyclase were maintained with Gly16 and Glu27 (4). On the other hand, the position 164 variant does not affect β_2AR down-regulation but is associated with lower agonist binding and lower activation of adenylate cyclase (5).

Clinically, genetic polymorphisms of β_2AR-16 and β_2AR-27 have been investigated from the viewpoint of the histopathologic diagnosis of asthma (6,7). The Gly16 allele has been shown to be associated with steroid-dependent and nocturnal asthma (2), but no such relationship was observed for the Gln27 allele (7). In asthmatic patients, Glu27 has been shown to be correlated with lower airway reactivity (8), and another study has indicated that Gln27 is associated with higher serum IgE levels in asthmatic families (9).

There have been many studies of the relationship between the β_2AR genotype and desensitization to β_2-agonists by means of the respiratory function test, but no definitive conclusions have been reached. The Gly16 variant has been shown to be associated with desensitization to β_2-agonists (6,10,11). A greater degree of bronchodilator desensitization was reported in the homozygote for Gly16 (GlyGly16) (10). In contrast, the deleterious response to regular inhaled β_2-agonist treatment was unrelated to β_2AR polymorphism, and forced expiratory volume in 1 second values induced by inhaled formoterol was not associated with the Gly16 allele (12,13).

The purpose of this study was to evaluate 1) the β_2AR genotype frequency in the Japanese population and 2) the relationship between β_2AR-16 genotype and desensitization to β_2-agonists *ex vivo*. The genotypes for β_2AR-16, β_2AR-27, and β_2AR-164 were determined in 92 healthy Japanese subjects by polymerase chain reaction (PCR)–restriction fragment length polymorphism. The relationship between the β_2AR-16 genotype and desensitization to β_2-agonists was examined in 10 male subjects *ex vivo*. Procaterol tablet (HCl salt, 50μg, Meptin®) was given orally for 5 days, and peripheral blood was obtained before and after 5 days of consecutive medications followed by assessment of the intracellular cAMP levels in peripheral blood mononuclear cells (PBMCs) after incubation with or without procaterol hydrochloride (0–1000 ng/mL).

MATERIALS AND METHODS

Materials

A DNA Extractor WB Kit for the isolation of genomic DNA was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). *Taq* DNA polymerase, *Takara Ex Taq*™, was obtained from Takara Shuzo Co. (Kyoto, Japan). PCR primers were synthesized by Nisshinbo Industries, Inc. (Tokyo, Japan). The PCR product, including positions 16 and 27, was generated using the forward primer β_2AR-16,27F (5′-

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GCCTTCTTGCTGGCACCCCAT-3′) and the reverse primer β_2AR-16,27R (5′-CAGACGCTCGAACTTGGC-CATG-3′) (6). To detect β_2AR-164 polymorphisms, PCR was performed with the forward primer β_2AR-164F (5′-GGAC TTTTGGCAACTTCTGG-3′) and the reverse primer β_2AR-164R (5′-ACGAAGACCATGATCACCAG-3′) (14). The restriction enzymes *Nco*I, *Ita*I, and *Mnl*I were purchased from Takara Shuzo Co., Boehringer Mannheim (GmbH, Germany), and MBI Fermentas (Vilnius, Lithuania), respectively. The DNA molecular weight marker, pUC18 *Hae*III digest, and procaterol hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). All other materials were of reagent grade and were obtained commercially.

For separation of PBMCs from EDTA-treated peripheral blood, Ficoll–Hypaque mixtures (Dainippon Pharmaceuticals Co., Ltd., Osaka, Japan) were used. Intracellular cAMP concentrations were measured with BIOTRAK™ cAMP EIA system (Amersham Pharmacia Biotech, Tokyo, Japan).

Subjects

A total of 92 healthy Japanese subjects participated in the β_2AR genotype frequency study (32 male and 60 females, 27.2±7.2 years of age, 162.9±7.5 cm height, 55.4±9.8 kg weight). Ten subjects of 92 participated in the study on the relationship between β_2AR-16 genotype and desensitization to β_2-agonists *ex vivo* (10 males: 26.0±3.0 years, 167.1±4.2 cm height, 62.4±9.9 kg weight). The aims of the study were fully explained to all subjects, and their written informed consent was obtained.

β_2AR Genotype Frequency in the Japanese Population

Isolation of Genomic DNA

Samples of 2 mL of peripheral blood were drawn into sampling tubes containing 3 mg of EDTA-2Na, $2H_2O$. Genomic DNA was extracted from 0.5 mL of whole blood in the presence of concentrated NaI and SDS with a DNA Extractor WB Kit (15). An equal volume of lysis solution containing 1 % Triton X-100 was added to 0.5 mL of blood, and the nuclei were isolated according to Buffone and Darlington. (16). Isolated nuclei were suspended in enzyme reaction solution containing 1 % SDS and digested with 0.8 mg/mL proteinase K to liberate DNA from nuclear proteins. After a 1-h incubation at 37°C, NaI was added to the nuclear lysate to a final concentration of 4.5 mol/L along with 0.4 % SDS, then isopropanol was added. The mixture was shaken until a white precipitate appeared. The precipitate was collected by centrifugation and washed with 40 % isopropanol and 70 % ethanol. Total genomic DNA was resuspended in 50 μL of Tris-EDTA buffer (pH 8.0) containing 10 mmol/L Tris-aminomethane and 1 mmol/L EDTA.

Determination of the β_2AR Genotype

PCR amplification of genomic DNA was performed in a final volume of 50 μL consisting of approximately 500 ng of DNA, 2.5 units of *Takara Ex Taq*™, 375 μM of each deoxyribonucleoside triphosphate, and 10 pmol of each primer in the reaction buffer. Amplification was performed by denaturation at 94°C for 2 min followed by 30 cycles at 94°C for 40 s, 64°C for 40 s, and 72°C for 50 s. The final extension period was extended to 5 min at 72°C. A programmable heat block (PROGRAM TEMP CONTROL SYSTEM PC-701, Astec Co., Fukuoka, Japan) was used to control the temperature.

PCR products of 168 bp (5 μL) were completely digested to detect the β_2AR-16 polymorphisms with 2 units of *Nco*I in the appropriate basal buffer (final volume, 10 μl) at 37°C for 1 h. *Nco*I cuts a 22-bp fragment from the 3′ end of the 168-bp PCR product from both alleles and an 18-bp fragment of the 5′ end from the Gly16 allele. The β_2AR-27 genotypes were identified using another aliquot of the same PCR product. One unit of *Ita*I was added to the basal buffer (final volume, 10 μL) at 37°C for 1 h. *Ita*I released 105- and 63-bp fragments from the PCR product of the Gln27 allele only. For detection of β_2AR-164, the amplified 358-bp PCR product was digested with 2 units of *Mnl*I at 37°C for 1 h.

After digestion using the restriction enzymes, the fragments were separated by 4% agarose gel electrophoresis, along with a DNA molecular weight marker, pUC18 *Hae*III digest, as a reference. The genotype was determined by restriction enzyme digestion and visualization under ultraviolet illumination after staining with ethidium bromide.

Relationship between β_2AR-16 Genotype and Desensitization to β_2-Agonists *ex Vivo*

β_2-Agonist Administration to Healthy Subjects

The relationship between the β_2AR-16 genotype and the desensitization to β_2-agonists was examined in 10 male subjects *ex vivo,* consisting of the homozygote for Arg16 (ArgArg16; n = 3), the heterozygote with Gly16 (ArgGly16, n = 4), and the homozygote for Gly16 (GlyGly16, n = 3). Nine subjects were GlnGln27, and one was GlnGlu27. Procaterol tablet (HCl salt, 50 μg, Meptin®, Otsuka Pharmaceuticals Co., Ltd., Tokyo, Japan) was given orally for 5 days once a day at 9 a.m. (Day 1–5). None of subjects received any medicine during this study. Peripheral blood was obtained before and after 5 days of consecutive medications on Day 0 and Day 6, respectively, followed by an assessment of desensitization to β_2-agonist using PBMCs separated from the peripheral blood.

Measurement of cAMP Levels in PBMCs

Desensitization to β_2-agonist was assessed using PBMCs because the mononuclear cell β-adrenoceptor-binding capacity is well correlated with that in the pulmonary system (17). PBMCs were separated from EDTA-treated peripheral blood, and the collected cells were washed with Hanks' balanced salt solution. The cells (1×10^5 cells) were incubated with or without procaterol hydrochloride (0, 0.1, 1, 10, 100, and 1000 ng/mL) in 180 μL of Hanks' balanced salt solution at 37°C for 20 min. After cell lysis, intracellular cAMP levels in PBMCs were measured by ELISA. cAMP levels in PBMCs are indicated as percentages of those in untreated control subjects. All assay procedures were performed on the day of blood sampling. Day-to-day variation in baseline levels of cAMP was negligible. Desensitization was assessed by comparison of cAMP levels prior to with those after 5 days of consecutive medications.

Statistical Analysis

The results are expressed as means ± standard deviation. The statistical significance of differences between groups was examined using paired *t* test. A *P* value less than 0.05 was considered significant.

RESULTS

β_2AR Genotype Frequency in the Japanese Population

Table I lists the β_2AR-16, 27, and 164 genotype frequencies in the total of 92 healthy Japanese subjects. Allele frequencies were Arg16:Gly16 = 46%:54%, Gln27:Glu27 = 92%: 8%, and Thr164:Ile164 = 100%: 0%. There was no gender effect on the frequencies.

As shown in Table II, none of the subjects were concomitantly positive for both ArgArg16 and Glu27 alleles. The highest frequencies of combinations between the β_2AR-16 and -27 genotypes were observed for matches of Gly16 variants (ArgGly16 and GlyGly16) and GlnGln27.

Relationship between β_2AR-16 Genotype and Desensitization to β_2-Agonists *ex Vivo*

The relationship between the β_2AR-16 genotype and desensitization to β_2-agonist was examined in 10 male subjects *ex vivo*. Procaterol tablet (HCl salt, 50μg, Meptin®) was given orally for 5 days. PBMCs were obtained before and after 5 days of consecutive medications, and their cAMP levels were compared after incubation with or without procaterol hydrochloride (0–1000 ng/mL) (Fig. 1).

In all β_2AR-16 genotypes (ArgArg16, ArgGly16, and GlyGly16), the cAMP level in PBMCs was increased by incubation with procaterol hydrochloride in a concentration-dependent manner, and the increase of cAMP levels on Day 6 were suppressed compared with Day 0, suggesting β_2AR down-regulation. The suppression of cAMP levels on Day 6 was more significant in GlyGly16 than ArgArg16. The cAMP levels on Day 6 were 86.7%, 76.8%, and 65.7% of Day 0 for ArgArg16, ArgGly16, and GlyGly16, respectively, when PBMCs were incubated with 1000 ng/mL of procaterol hydrocloride.

DISCUSSION

In healthy Japanese subjects, the Gly16 allele was found at the same frequency as the Arg16 allele, and the Gln27 allele was found in 92% of the total population (Table I). The frequency of the Ile164 allele was less than 1% (Table I). In Caucasians, it was reported that the genotype frequencies in asthmatic patients were not different from the healthy subjects (18). The Gly16 allele was reported to be more common with the frequency of about 14%, 31%, and 55% for ArgArg16, ArgGly16, and GlyGly16, respectively, and the Gln27 and Glu27 allele frequencies were equivalent with the frequency of about 27%, 47%, and 26% for GlnGln27, GlnGlu27, and GluGlu27, respectively (18). The ThrIle164 genotype represented about 5%, but the homozygous Ile164 genotype was not found (18). In the majority of Japanese subjects, the β_2AR-16 mutation (ArgGly16 and GlyGly16) was accompanied with the wild-type β_2AR-27 gene (GlnGln27) (Table II), whereas no such relationship was found in Caucasians (7,9,18–20).

In this study, the relationship between β_2AR-16 genotype and desensitization to β_2-agonist was also evaluated. Despite the β_2AR-16 genotype, the cAMP levels were increased by incubation with procaterol hydrochloride, and the increase was suppressed after 5 days of consecutive medications, suggesting β_2AR down-regulation. The suppression was more significant in the homozygote for Gly16 than the homozygote for Arg16 (Fig. 1). This was not contradictory to *in vitro* findings; that is, the Gly16 variant was associated with enhanced β_2-agonist-induced receptor down-regulation (2–4). Subjects given procaterol orally were ArgArg16 (n = 3), ArgGly16 (n = 4), and GlyGly16 (n = 3). Nine subjects were GlnGln27, and one was GlnGlu27. Although it is still not known whether the Glu27 allele participates in β_2AR down-regulation *in vivo,* the transfected cells co-expressing Gly16 and Glu27 displayed a greater degree of down-regulation as compared with the wild type (4), and this genetic contamination seemed to have no effect on the relationship between β_2AR genotype and β_2-agonist desensitization *ex vivo*. The down-regulation was confirmed only for 5 days of consecutive

Table I. β_2AR-16, -27, and -164 Genotype Frequency in Healthy Japanese Subjects

Genotype	Male	Female	Total
ArgArg16	7 (21.9)	12 (20.0)	19 (20.7)
ArgGly16	19 (59.4)	27 (45.0)	46 (50.0)
GlyGly16	6 (18.8)	21 (35.0)	27 (29.3)
GlnGln27	28 (87.5)	52 (86.7)	80 (87.0)
GlnGlu27	4 (12.5)	6 (10.0)	10 (10.9)
GluGlu27	0 (0.0)	2 (3.3)	2 (2.2)
ThrThr164	32 (100.0)	58 (100.0)	90 (100.0)
ThrIle164	0 (0.0)	0 (0.0)	0 (0.0)
IleIle164	0 (0.0)	0 (0.0)	0 (0.0)

Numbers in parentheses are percentages within each group.

Table II. Association between β_2AR-16 and -27 Genotypes in Healthy Japanese Subjects

Genotype	GlnGln27	GlnGlu27	GluGlu27	Total
ArgArg16	19 (20.7)	0 (0.0)	0 (0.0)	19 (20.7)
ArgGly16	42 (45.6)	3 (3.3)	1 (1.1)	46 (50.0)
GlyGly16	19 (20.7)	7 (7.6)	1 (1.1)	27 (29.3)
Total	80 (87.0)	10 (10.9)	2 (2.2)	92 (100)

Values are presented as the number of subjects with percentages in parentheses.

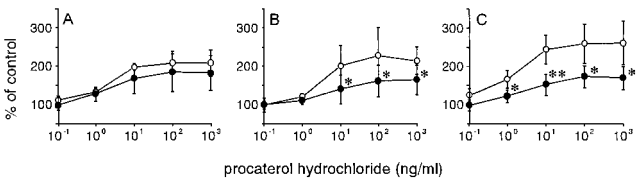

Fig. 1. Efects of procaterol hydrochloride (0–1000 ng/mL) on cAMP levels in peripheral blood mononuclear cells on Day 0 (○) and on Day 6 (●) after 5 days of consecutive medications of procaterol tablet (HCl salt, 50 μg, Meptin®). A–C indicate the data for ArgArg16 (n = 3). ArgGly16 (n = 4) and GlyGly16 (n = 3), respectively. Each circle represents the mean ± standard deviation. *·**Statistically significant compared with Day 0: $P < 0.05$ and 0.01, respectively.

medications, but long-term medication is conducted in regular β_2-agonist therapy, suggesting extensive down-regulation might be associated with the Gly16 variant. In this context, cAMP was used as the indicator, and further investigations should be performed concerning agonist binding and coupling to adenylate cyclase *ex vivo*.

A number of proteins have been found to show genetic polymorphisms, and investigations have focused on genetic diagnosis or the pathogenesis of genetic diseases. Recent findings of genetic polymorphisms in drug metabolizing enzymes, including NAT2 (21,22), cytochrome P450 (CYP) 2C9, CYP2C19 (23–25), and CYP2D6 have suggested that genotyping should be performed to establish individual dosage regimens. Procaterol is rapidly absorbed after oral administration and metabolized in the liver, so it is likely that genetic differences in many hepatic enzymes are related to the interindividual variability of the bronchodilating action of this drug. In our preliminary study, however, CYP2C9 and CYP2C19 contributed little to the metabolism of procaterol, suggesting there was relatively small variation of procaterol pharnmacokinetics. This was indicated by small variation in baseline cAMP levels on Day 6 (data was not shown).

In conclusion, desensitization to β_2-agonist was associated more frequently with the mutation at β_2AR-16 (Gly16). As many asthmatics use β_2-agonists over long periods, it is important to understand the β_2AR genetic factors determining the responses to asthma therapy, and the medication after prediction by operating genotyping may be useful for establishing the optimal dosage regimen for each patient and for preventing adverse reactions.

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