Effects of Ile164 Polymorphism of Beta₂-Adrenergic Receptor Gene on Coronary Artery Disease

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Objectives

This study investigated the relationship between beta₂-adrenergic receptor (B2AR) Ile164 polymorphism and coronary artery disease (CAD).

Background

B2ARs are crucial to the regulation of vascular tone, and neoangiogenesis is impaired in the presence of isoleucine at position 164 (Ile164) B2AR gene polymorphism. No data deal with the role of the variants at position 164 of the B2AR gene in the setting of CAD.

Methods

The study population consisted of 330 patients undergoing percutaneous coronary intervention (PCI).

Results

The Ile164 polymorphism frequency was higher in CAD (12.1% vs. 3%, p \( \leq 0.008 \)) with respect to the control population. We divided our population into 2 groups: group 1 (290 patients, threonine/threonine genotype at position 164 [164Thr/Thr]) and group 2 (40 patients, threonine/isoleucine genotype at position 164 [164Thr/Ile]). Patients of group 2 presented an earlier onset of CAD (56.7 ± 7.8 vs. 59.5 ± 10, p = 0.04) and a higher incidence of multivessel disease (25.4% vs. 41%, p = 0.044). At follow-up, group 2 showed a higher incidence of new acute myocardial infarction (17.5% vs. 4.5%, p = 0.001), new PCI (37.5% vs. 13.1%, p = 0.0001), and cardiac death (10% vs. 3.1%, p = 0.036). Cox regression analysis identified Ile164 as an independent predictor of cardiac death (odds ratio [OR]: 3.731, 95% confidence interval [CI]: 1.004 to 13.867, p = 0.049) and an overall major adverse cardiac event (OR: 4.100, 95% CI: 1.945 to 8.640, p = 0.0001). A replication study was done on a population of 150 patients with peripheral artery disease. The presence of the Ile164 allele was associated with a higher incidence of acute myocardial infarction (54.5% vs. 25.2%, p = 0.035) or combined events (acute myocardial infarction, PCI, or coronary artery bypass graft) (63.6% vs. 30.9%, p = 0.027).

Conclusions

Our study suggests that the B2AR Ile164 mutant is associated with a more aggressive CAD and adversely affects prognosis in patients undergoing PCI. (J Am Coll Cardiol 2008;52:1381–8) © 2008 by the American College of Cardiology Foundation

The sequence of human beta₂-adrenergic receptor (B2AR) is highly polymorphic, and there are 3 major coding sequence polymorphisms: arginine at position 16 replaced by glycine (Arg16Gly), glutamine at position 27 replaced by glutamic acid (Gln27Glu), and threonine at position 164 replaced by isoleucine (ThrIle164) (1). The functional relevance of these variants has been extensively evaluated in cells (2–4) and in transgenic mice (5) and is mostly related to cardiac function (2,3). In particular, the isoleucine (Ile164) substitution of the threonine in position 164 causes that receptor to have decreased basal and agonist-stimulated adenylyl cyclase activities and decreased affinity for B2AR agonists (6). Taking into consideration the pathophysiological consequences of polymorphic B2AR, it is important to note that B2ARs are highly expressed throughout the cardiovascular system, in which they mediate increased myocardial inotropism and chronotropism (7,8) and regulate coronary vasodilation (9). Recently, we have demonstrated that the endothelial B2AR contributes to vasorelaxation and to post-ischemic angiogenesis (10). Interestingly, the Ile164 variant of the B2AR appears to lose the ability to modulate such responses. Similarly, vasodilation in humans carrying the Ile164 variant of the B2AR gene is impaired (6). To date, no data deal with the role of the Ile164 variant of the B2AR gene in the setting of coronary artery disease (CAD). It is well known that CAD is not a consequence of...
either a single gene or just 1 environmental factor, but rather it is a complex, multifactorial disease. Moreover, the field of B2AR polymorphism research, particularly with regard to clinical associations, is characterized by rather controversial data being reported. Overall, the available clinical association data have proved to be rather disappointing as most originally proposed relationships have not been consistently replicated in later studies (11,12). This study was designed to investigate the relationship between B2AR Ile164 polymorphism and CAD.

Methods

In Vivo Study

Patients. The study population consisted of 330 consecutive patients (mean age 60 ± 11 years, 84% male) undergoing elective or urgent percutaneous coronary intervention (PCI) for CAD documented by a positive stress test or by T1 single photon emission computed tomography or acute coronary syndrome. At the time of PCI, peripheral blood was drawn to determine the B2AR genotype. To identify normal distribution of the B2AR genotype, we enrolled a control population of 100 gender-matched healthy unrelated individuals (58 ± 10 years, 80% male), recruited from blood donors of our blood bank. Controls were free from heart disease, medication use, and cardiovascular risk factors, except for smoking habits. A replication analysis was performed on an independent population of the PACE (Prevention by Low Dose Aspirin of Cardiovascular Disease in the Elderly) study, which was included in a cardiovascular risk assessment in older adults. The study included patients (mean age 67 ± 8 years, 80% male) recruited from blood donors of our blood bank. Patients (mean age 67 ± 8 years) were drawn during a scheduled follow-up visit. A written informed consent was obtained from all patients according to the Ethics Committee of the Federico II University of Naples School of Medicine.

PCI procedure. PCI was performed according to the American Heart Association/American College of Cardiology guidelines (14). Antegrade perfusion was graded by Thrombolysis In Myocardial Infarction (TIMI) criteria (15). Angiographic lesion morphology was classified according to American Heart Association/American College of Cardiology classification (16). Stenoses >50% were considered significant. All patients received bare-metal stents to optimize acute and long-term procedural results. Glycoprotein IIb/IIIa inhibitors were used in 30% of patients. After the procedures, all patients were on dual antiplatelet therapy with aspirin and clopidogrel for 30 days. Long-term medical treatment was left to the discretion of the attending cardiologists.

Follow-up. In the evaluation of long-term clinical outcome, major adverse cardiac events (MACE) were considered as end points, including cardiac death, acute myocardial infarction (AMI), new PCI, bypass cardiac surgery, and development of congestive heart failure. The follow-up was based on a direct systematic review of all patients' clinical files for a mean study period of 36 ± 4 months, contacting relatives or a patient's physician when necessary. Follow-up was completed in all patients and data stored in a computerized database.

Carotid ultrasound analysis. Twelve patients (6 with B2AR Ile164 and 6 with B2AR threonine in position 164 [Thr164]) were selected to be within 1 SD of the average for age and risk factors for atherosclerosis. A carotid ultrasound examination was performed using a 7.5-MHz, linear-array transducer (SONOS-5500, Philips Healthcare, Andover, Massachusetts) as previously described (17). The operator performed all of the carotid scans without any information on the genotype. Intima-media thickness (IMT) was defined as the distance from the leading edge of the lumen-intima interface to the leading edge of the media-adventitia interface. Atherosclerotic plaque was defined as a lesion with focal IMT of 1.5 mm or more with a localized protrusion of the vessel wall into the lumen. Integrated backscatter (IBS) values of all carotid atherosclerotic plaques were measured as described previously (18), expressing the relative IBS value of the intima-media complex as the difference in IBS values between the intima-media and adventitia.

B2AR genotyping. Using a commercially available kit (Midiprep DNA, Qiagen, Valencia, California), genomic deoxyribonucleic acid (DNA) was isolated from 2 ml of the peripheral blood samples. The Ile164 B2AR gene polymorphism was studied using combined polymerase chain reaction (PCR) and restriction fragment length polymorphism technique on PCR amplified products using PCR conditions and primers on a Thermocycler (MJ Research, St. Bruno, Canada) and DNA polymerase (TAQ, Qiagen) (19).

In Vitro Study

Adenoviral mediated gene transfer of the B2AR gene in vascular smooth muscle cells. We used adenoviral vectors encoding for the human B2AR wild-type (WT) gene and the B2AR-Ile164 mutant gene as previously reported (10,20,21). Cells were infected at a multiplicity of infection of 100 plaque-forming units per cell, in the presence of serum for 3 h at 37°C.
[\textsuperscript{3}H]-thymidine incorporation. Vascular smooth muscle cells (VSMCs) were starved for 24 h and then incubated in Dulbecco modified Eagle medium with [\textsuperscript{3}H] thymidine and beta-AR agonist isoproterenol (ISO) (10\textsuperscript{–7} mol/l). After 24 h, thymidine incorporation was evaluated as described elsewhere (22).

Cell culture and proliferation. Arterial VSMCs were obtained from rat aorta by enzymatic digestion, as described elsewhere (23). Cells were grown in plastic dishes in medium 199 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and studied between passages 4 and 10. The VSMCs were seeded at a density of 10,000 per well in 6-well plates, serum starved overnight, and then stimulated with ISO. Cell numbers were studied at 6, 12, and 24 h after stimulation with ISO 10\textsuperscript{–7} mol/l. At any of the time points, medium was removed, detached from plate by 3 min trypsin incubation, and counted on a hemocytometer.

B2AR density and membrane adenyl cyclase activity assays. Crude cell membranes were prepared as previously described. The B2AR density was determined by radioligand binding with the nonselective B2AR ligand 125I-labeled iodocyanopindolol using standard methods (24).

Western blot. The VSMCs were stimulated with ISO for 5 min for extracellular signal-regulated kinase (ERK) or up to 9 h for phosphorylated retinoblastoma protein (Rb) and then dissolved in radioimmunoprecipitation assay–sodium dodecylsulfate buffer. Protein were electrophoresed by sodium dodecylsulfate/polyacrylamide gel electrophoresis and transferred to nitrocellulose; Rb, phosphorylated ERK1/2, total ERK (Cell Signaling Technology, Danvers, Massachusetts), and actin (Santa Cruz Biotechnology, Santa Cruz, California) were visualized by specific antibodies, antirabbit horseradish peroxidase–conjugated secondary antibody (Santa Cruz) and standard chemiluminescence (Pierce Biotechnology, Rockford, Illinois) on autoradiographic films. Autoradiographs were then digitalized and densitometry quantification performed using dedicated software (ImageQuant, Molecular Dynamics, Sunnyvale, California). Data are presented as arbitrary densitometry units after normalization for the total corresponding protein or actin as internal control.

Statistical analysis. Continuous variables are presented as mean ± SD and categorical variables as absolute number and percentage value. Differences between groups were assessed using univariate analysis of variance for continuous variables, with a Bonferroni post-hoc test for evaluation of multiple comparisons. Categorical variables were analyzed by chi-square test, and odds ratio (OR) with 95% confidence intervals (CI) or by the Fisher exact test when the expected values in any of the cells of the test, given the frequencies and the overall sample size, was below 10; p value <0.05 was considered significant. Difference in event-free survival between groups were evaluated by the Kaplan-Meier method, comparisons were made using log-rank test. A Cox regression analysis was conducted for the 164 position B2AR polymorphism considering age, cardiovascular risk factors, medication use, left ventricular ejection fraction, angiographic characteristics, and interaction of the 164th position B2AR polymorphism in both the study populations. The computer program used was SPSS 12.1 (SPSS Inc., Chicago, Illinois).

Results

Thr\textsuperscript{Ile}164 frequencies. In line with previously reported data, we could not find homozygous Ile164 individuals in studies and control groups. Of note, the frequency of the Ile164 allele was significantly higher in the CAD population as compared with the healthy population (12.1% vs. 3%, respectively, p = 0.008). We, therefore, focused on the effects of Ile164 polymorphism on clinical, angiographic, and prognostic characteristics of CAD. The study population was divided into 2 groups: group 1 (290 patients, threonine/threonine genotype at position 164 [164Thr/Thr]; 86% male, mean age 60 ± 10 years) and group 2 (40 patients, threonine/isoleucine genotype at position 164 [164Thr/Ile]; 83% male, mean age 60 ± 8.57 years).

Population clinical characteristics. There were no differences between groups with regard to clinical features (Table 1). Accordingly, the preprocedural left ventricular ejection fraction was similar between groups (46.3 ± 8% vs. 49.4 ± 10%, p = NS). However, when we considered the age when CAD was first diagnosed, patients of group 2 were significantly younger, thus suggesting an earlier onset of CAD (56.7 ± 8 years vs. 59.6 ± 10 years, p = 0.04) in patients harboring the Ile164 polymorphism.

Population angiographic characteristics. Group 2 presented more severe angiographic CAD as indicated by the higher incidence of multivessel disease, and by a larger occurrence of type B2/C lesions (Table 2). Accordingly, the
number of vessels treated by PCI was significantly higher in group 2.

**Medication use.** There were no differences between groups regarding medication use at the time of PCI and during follow-up. In particular, at follow-up time both groups were similarly treated, using double antiplatelets therapy (99% vs. 98% group 2, p = NS), calcium antagonist (19% vs. 18% group 2, p = NS), beta-blockers (45% vs. 47% group 2, p = NS), angiotensin-converting enzyme inhibitors (36% vs. 34% group 2, p = NS), and statins (42% vs. 40% group 2, p = NS).

**Long-term follow-up.** There were no significant differences between groups regarding MACE incidence during in-hospital stay. At long-term follow-up, the rate of PCI (37.5% vs. 13.1%, p < 0.0001), and development of heart failure (12.5% vs. 2.1%, p = 0.001) were significantly higher in group 2. Furthermore, patients harboring the Ile164 allele showed a significantly increased rate of new AMI (17.5% vs. 4.5%, p = 0.001) and cardiac death (10% vs. 3.1%, p = 0.036). Accordingly, the Kaplan-Meier analysis for event-free survival in patients harboring the Ile164 allele (Fig. 1) showed worse long-term outcome for these patients (p = 0.0001). Cox regression analysis identified the presence of Ile164 as an independent predictor for cardiac death (OR: 0.2, p = 0.035) or combined events (AMI, PCI, or coronary artery bypass graft) (63.6% vs. 30.9%, p = 0.027). Indeed, in this population, the Ile164 allele increased the risk of severe cardiovascular clinical outcome as confirmed by Cox regression analysis after correction for multiple covariates (OR: 7.803, 95% CI: 1.358 to 5.730, p = 0.005).

**Carotid ultrasound analysis.** Carotid ultrasound analysis was performed during hospitalization. Patients harboring the Ile164 polymorphism presented a significantly increased amount of atherosclerotic plaque in the carotid vascular district (1.3 ± 0.1 vs. 0.6 ± 0.2, p = 0.035). Accordingly, IMT was significantly higher in these patients (Fig. 2A). Interestingly, plaque analysis performed by correct IBS technique suggests a different plaque composition between groups with a higher presence of “soft tissue” in Ile164 patients (Fig. 2B). These data suggest a more aggressive development of atherosclerosis in presence of the Ile164 gene polymorphism.

**In vitro data.** We have previously demonstrated that the Ile164 variant of the B2AR gene in endothelial cells loses the ability to mediate cell specific responses to catecholamine (10,22). Here, to gain better insight on the role of Ile164 on atherosclerosis, we explored the effect of this polymorphism on VSMC proliferation in culture. In subconfluent rat aorta VSMC, the ISO caused a significant increase in cell number after 24 h (Fig. 3A). Consistent with an effect on cell proliferation, beta-AR stimulation with ISO resulted in an increase in [3H]-thymidine incorporation (Fig. 3B), an index of DNA synthesis, and phosphorylation of Rb protein, indicative of progression in the cell cycle (Figs. 3C and 3D). Moreover, we found that ISO leads to significant ERK activation. To amplify the B2AR signal and better dissect the effect of the B2AR Ile164 polymorphism on biological responses of VSMC, we in-

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**Table 2 Angiographic Characteristics of B2AR Ile164 Patients**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group 1 (n = 290)</th>
<th>Group 2 (n = 40)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary artery disease, %</td>
<td>60.6</td>
<td>53.8</td>
<td>NS</td>
</tr>
<tr>
<td>CX</td>
<td>69.3</td>
<td>35.9</td>
<td>NS</td>
</tr>
<tr>
<td>DX</td>
<td>33.1</td>
<td>59</td>
<td>0.002</td>
</tr>
<tr>
<td>Lesion type B2/C, %</td>
<td>75.5</td>
<td>100</td>
<td>0.048</td>
</tr>
<tr>
<td>More than 1 vessel treated, %</td>
<td>21.6</td>
<td>42.5</td>
<td>0.005</td>
</tr>
<tr>
<td>Multivessel disease, %</td>
<td>25.4</td>
<td>41</td>
<td>0.044</td>
</tr>
<tr>
<td>TIMI flow grade 3 after PCI, %</td>
<td>97</td>
<td>96.5</td>
<td>NS</td>
</tr>
</tbody>
</table>

CX = left circumflex coronary artery; DX = right coronary artery; LAD = left anterior descending coronary artery; TIMI = Thrombolysis in Myocardial Infarction; other abbreviations as in Table 1.
results in a strong independent predictor of worse prognosis at long-term follow-up. To identify the possible mechanism, we investigated the nature of atherosclerotic plaque by IBS ultrasound and found peculiarities of the carotid atherosclerosis plaque in patients harboring Ile164 polymorphism. These differences can be explained by a different content in VSMCs, which are less prone to proliferation in Ile164 B2AR patients. Our corollary data support a role for this polymorphism on the pathogenesis of a plaque that has a different composition due to a defect in VSMC proliferation.

**Early and diffuse atherosclerosis in Ile164 subjects.** We found an association between CAD onset age and the presence of B2AR Ile164. Although there were no significant differences in mean age between groups (60.10 vs. 60.8 years in group 2, p = NS), when we compared the age at onset of CAD, we found a significantly younger age in group 2 (56.7 vs. 59.5 years, p = 0.04), suggesting an earlier development of CAD. Atherosclerosis is a diffuse process that starts early in childhood and progresses asymptotically through adult life affecting different vascular territories (25). Accordingly, we found a disease involving different vascular districts. The presence of a severe CAD was angiographically confirmed by the observation of a higher incidence of type B2/C lesions, a larger occurrence of multivessel disease, and a significantly higher number of severe lesions needing treatment by PCI. Interestingly, we found a significantly higher number of atherosclerotic plaque in patients harboring the Ile164 with a higher IMT value. Therefore, data on carotid and coronary arteries suggest an association between the Ile164 polymorphism of the B2AR gene and the development of a severe and diffuse atherosclerosis.

**Poor outcome in B2AR Ile164 subjects.** In our study, we found a strong association between Ile164 and prognosis. Indeed, overall MACE were significantly higher in patients with the Ile164 variant compared with the WT receptor. Despite the wide use of coronary stenting and the optimal result of PCI, with a similar rate of TIMI antegrade flow grade 3 after procedure, group 2 showed a significant higher prevalence of adverse clinical events with a higher incidence of new PCI, new AMI, heart failure, and cardiac death. Cox regression analysis identified the presence of the Ile164 polymorphism as a strong independent risk factor for adverse clinical outcomes associated with a higher risk for cardiac death. The association between Ile164 and atherosclerosis was confirmed in a replication study. In an independent population of patients affected by PAD, and therefore at risk for CAD, the Ile164 polymorphism prevalence, although not significant, was higher than in the present control population and almost all previously published control populations (1,6). Moreover, those harboring the B2AR Ile164 polymorphism showed a 3.6 X increased risk to develop AMI and a 3.9 X increased risk to develop a major cardiovascular event. These data confirm the prognostic role of the B2AR’s 164 position in cardiovascular disease.
Potential mechanism. The proliferation of VSMCs is a key event in the formation of atherosclerotic lesions and the development of restenosis following angioplasty (26–28). Studies in coronary atherectomy specimens showed that in patients with unstable plaque, the numbers of inflammatory cells increased, whereas the number of VSMCs decreased reciprocally to the severity of the clinical presentation of CAD (29). In this study, we found that VSMC proliferation is impaired in patients carrying the Ile164 polymorphism as shown by reduced cell proliferation, [3H]-thymidine incorporation and Rb phosphorylation. We can speculate that a dysfunctional B2AR leads to a reduced cellularity in atherosclerotic plaques due to a reduction in VSMC proliferation leading to development of plaques more prone to instability and rupture. Accordingly, patients with Ile164 polymorphism showed a 4.5× increased risk of developing a new AMI. Our hypothesis is further supported by the IBS analysis of carotid arteries plaques. Measurement of IBS to evaluate plaque composition is an effective ultrasonic...
technique to distinguish atheromatous (fibrofatty lesion with a lower IBS) tissue from fibrous tissue (fibrocalcific lesion with a higher IBS) (30), and carotid IMT by ultrasound was recently reported as a good surrogate marker of coronary atherosclerosis (31). We found in vivo a significantly higher incidence of lower-IBS lesions in the carotid arteries of patients with Ile164 polymorphism, which is consistent with the presence of atherosclerotic plaque with a lower VSMC component in patients carrying this gene polymorphism. Moreover, these patients presented a significantly higher incidence of a new PCI. Because VSMCs are the sole producers of the tensile components of the extracellular matrix (notably of collagen type I), a decrease in their numbers would lead to diminished production of the plaque-stabilizing molecules and, therefore, to reduced plaque stability. Thus, we can speculate that alteration in vessel biology, namely VSMC proliferation and/or endothelial cell turn over, can be responsible for these phenomena leading to a very poor clinical outcome for patients.

Study limitations. Limitations of the study include those inherent to any prospective but observational study. Because this is an association study, we cannot rule out the presence of a possible linkage disequilibrium with other neighboring genes that might explain the significant association with atherosclerotic phenotype or adverse prognosis. The study was conducted in patients with severe CAD undergoing PCI and the replication study was performed in patients with PAD; therefore, our findings need to be confirmed in further larger patient populations. Nevertheless, a recent study (32) underlines the need for selection of patients in association studies in order to identify the populations in which single gene polymorphisms may be more determinant in complex phenotypes such as CAD. Finally, all patients received bare metal stents during PCI; therefore, we cannot exclude a reduced incidence of new PCI at long-term follow-up using drug eluting stents. However, the efficacy and safety of the use of drug eluting stents on long-term outcome remains to be established and, in the recent ERACI III (Randomized Trial of Percutaneous Transluminal Coronary Angioplasty Versus Coronary Artery Bypass Surgery in Multivessel Disease) study (33), the initial advantage over bare metal stents appeared to decrease with time.

Conclusions and Clinical Implications

Our study suggests that the B2AR-Ile164 mutant is associated with an earlier and more aggressive CAD, and it adversely affects prognosis in patients with severe CAD undergoing PCI. These findings confirm the pivotal role of B2AR function in the cardiovascular system and add new evidence to existing data on the linkage between B2AR function and outcome in heart disease.

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Key Words: beta2-adrenergic receptor • single nucleotide polymorphism • vasodilation.