Risk of Coronary Artery Disease Associated With Polymorphism of the Cytochrome P450 Epoxygenase CYP2J2

Martin Spiecker, MD; Harald Darius, MD; Thomas Hankeln, PhD; Muhidien Soufi, PhD; Alexander M. Sattler, MD; Jürgen R. Schaefer, MD; Koichi Node, MD; Jan Börgel, MD; Andreas Mügge, MD; Klaus Lindpaintner, MD; Anika Huesing, MSc; Bernhard Maisch, MD; Darryl C. Zeldin, MD; James K. Liao, MD

Background—Cytochrome P450 (CYP) 2J2 is expressed in the vascular endothelium and metabolizes arachidonic acid to biologically active epoxyeicosatrienoic acids (EETs). The EETs are potent endogenous vasodilators and inhibitors of vascular inflammation. However, it is not known whether genetic polymorphisms of CYP2J2 are associated with increased cardiovascular risks.

Methods and Results—All 9 exons of the CYP2J2 gene and its proximal promoter were sequenced in 132 patients to identify potential variants. Functional consequence of a single nucleotide polymorphism (SNP) in the promoter of CYP2J2 was further evaluated by use of transcription factor–binding and reporter assays. A total of 17 polymorphisms were identified. One of the most relevant polymorphisms in terms of frequency and functional importance is located at /H11002 (G-50T) in the proximal promoter of CYP2J2. Screening of 289 patients with coronary artery disease and 255 control subjects revealed 77 individuals with the G-50T SNP (17.3% of coronary artery disease patients, 10.6% of control subjects; P = 0.026). The association of the G-50T polymorphism remained significant after adjustment for age, gender, and conventional cardiovascular risk factors (OR, 2.23; 95% CI, 1.04 to 4.79). The G-50T mutation resulted in the loss of binding of the Sp1 transcription factor to the CYP2J2 promoter and resulted in a 48.1 ± 2.4% decrease in CYP2J2 promoter activity (P < 0.01). Plasma concentrations of stable EET metabolites were significantly lower in individuals with the G-50T SNP.

Conclusions—A functionally relevant polymorphism of the CYP2J2 gene is independently associated with an increased risk of coronary artery disease. (Circulation. 2004;110:2132-2136.)

Key Words: atherosclerosis ■ coronary disease ■ genetics ■ inflammation ■ molecular biology

Cytochrome P450 epoxygenases metabolize arachidonic acid to epoxyeicosatrienoic acids (EETs). The human cytochrome P450 enzyme, CYP2J2, is abundantly expressed in coronary artery endothelial and smooth muscle cells and in cardiac myocytes.1,2 One of the primary products of the NADPH-dependent epoxidation of arachidonic acid by CYP2J2 is the production of 11,12-EET. This eicosanoid exerts antiinflammatory effects by inhibiting endothelial nuclear factor-κB, a transcription factor that plays a key role in the induction of many proinflammatory gene products in the vascular wall.2,3 Additionally, 5,6-, 8,9-, 11,12-, and 14,15-EETs have important vasodilator properties via hyperpolarization and relaxation of vascular smooth muscle cells.4–6 Recently, additional vascular protective effects of EETs, including antithrombotic, antimigratory, antioxidant, and antiapoptotic effects, have been observed.7,8 A large degree of interindividual variation in CYP2J2 expression has been observed.1 Among the factors known to be associated with differential human P450 gene expression are genetic polymorphisms.9,10 Given the potential importance of CYP2J2 in vascular function, we investigated whether genetic variants of this novel gene are associated with cardiovascular disease and, if so, to determine the potential mechanism involved.

Methods

Study Population
We studied 544 unrelated male and female white subjects, including 289 cases of coronary artery disease (CAD), as defined by angio-
graphically documented coronary artery stenosis of >50% severity. Control patients (n=235) had no coronary vessels with >50% stenosis. The studied subjects were consecutive patients with an age limit of 65 years encountered in the cardiac catheterization laboratory from August 1998 to July 2000 and from October 2001 to August 2002. All participants came from central Germany and had comparable socioeconomic backgrounds. All patients gave informed consent that explicitly provided permission for DNA analysis and collection of relevant clinical data. Whole blood (10 mL) was collected in EDTA-anticoagulant tubes by phlebotomy. At the same time, a complete clinical history, including cardiovascular risk factors, was obtained from all study subjects. Criteria for the diagnosis of myocardial infarction were chest pain lasting >30 minutes, elevated cardiac enzymes, and ECG changes suggestive of myocardial infarction. Diagnosis of stroke or transient ischemic attack was based on the findings of physical examination by a neurologist and confirmed by cranial CT. The Institutional Review Board approved the study protocol.

Polymorphism Analysis
Patient DNA was isolated from whole-blood samples by the phenol-chloroform extraction method. In the first 132 patients (78 with CAD, 54 without CAD), all 9 exons, intron-exon boundaries, and the proximal promoter of CYP2J2 were analyzed for genetic variants by polymerase chain reaction (PCR) and direct sequencing of PCR products. A total of 4613 base pairs (bp) were screened. Primers, size of amplification products, and PCR conditions used for amplification of CYP2J2 exons have been described previously by King et al.11 A 273-bp promoter region proximal to the transcriptional start site was amplified with the exon 1 primers described by King et al.11 The sequence reaction used dye terminator chemistry (Applied Biosystems), and the sequence products were resolved on an ABI 377 automated sequencer. The promoter polymorphism G-50T was further verified by direct sequencing in 154 patients with angiographically documented CAD and in 145 patients without CAD. The numbering of the polymorphisms refers to the GenBank sequence H11022.

Subsequently, genotype identification by a restriction endonuclease digestion system was established and used for further genotype analysis in the remaining 245 patients. The G-50T polymorphism creates a novel restriction site for the Alul endonuclease (recognition sequence AGCT). PCR (using the following primers: sense, 5'-TTTTCTGAGACCGGTGCTGCTG-3'; antisense, 5'-TAGGAGACTTTTTCTGAGACCGGTGCGTG-3') yielded a 242-bp product. Incubation with Alul resulted in 2 fragments (99 and 143 bp) in PCR products with the G-50T allele. Single nucleotide polymorphism (SNP) genotyping using Kpn I and Xho I endonuclease (recognition sequences Kpn I and Xho I) yielded 2 fragments (99 and 143 bp) in PCR products with the G-50T allele.

Results
Sequencing of the CYP2J2 gene in 132 white patients revealed several genetic variants in the promoter region, in exons 1, 2, 4, 6, and 9; in intronic regions; and in the 5'-untranslated region (Table 1). Among them, 3 rare (frequency <0.01) polymorphisms in exons 2, 4, and 9 are predictive of amino acid substitutions in the CYP2J2 protein. More frequent polymorphisms in exons 1 and 6 were silent and therefore not considered to be of clinical importance. An SNP affecting the promoter region at position -50 (relative to the transcriptional start site), representing a substitution of a guanidine by a thymidine nucleotide (G-50T), was identified. The TT genotype was found in 1.5% and the GT genotype in 11.4% of patients. Given the prominent fre-
Analysis of the CYP2J2 promoter with G-50T polymorphism revealed the loss of binding site for the transcription factor Sp1 (Figure 1). We performed DNA binding studies to assess the functional relevance. Using a G→T mutated oligonucleotide in electrophoretic mobility shift assay studies, we reduced Sp1 binding significantly compared with WT oligonucleotide (Figure 2A). Addition of unlabeled oligonucleotide abolished the Sp1 binding, and preincubation with an Sp1 antibody supershifted the Sp1 band, confirming the specificity of the Sp1-DNA binding (data not shown). To determine the functional consequences of reduced Sp1-DNA binding on transcriptional activation of the CYP2J2 gene, transfection studies with promoter-reporter gene constructs were performed. In nonstimulated endothelial cells, the construct containing the WT promoter induced a 42-fold increase in promoter activity compared with the empty pGL2 vector (Figure 2B). The basal activity of the G-50T mutant promoter, however, was reduced by 48±2% (P<0.01) compared with the WT construct. Preincubation of transfected cells with tumor necrosis factor-α, interleukin-1, nifedipine, cortisol, and diclofenac had no effect on basal WT or mutant CYP2J2 promoter activity (data not shown).

To further investigate the functional role of the G-50T polymorphism, we measured the plasma concentrations of the major CYP2J2-dependent epoxidation product from arachidonic acid. Given the instability of the primary products, EETs, concentration of the stable metabolite 14,15-DHET was determined after extraction from plasma samples (Figure 3). Median DHET plasma concentrations were significantly lower in samples from individuals with the G-50T polymorphism (5.25 ng/mL) compared with WT individuals (7.4 ng/mL; P=0.028).

Given these functional findings, we analyzed the frequency of GT and TT genotypes in 289 patients with angiographically documented CAD and 255 control subjects with angiographic exclusion of CAD. The G-50T (GT and TT genotype) SNP was found in 10.6% of control subjects and 17.3% of CAD patients. The GT genotype was present in 10.2% of control subjects and 14.9% of CAD patients; the TT genotype was found in only 0.4% of control subjects and 2.4% of CAD patients. Considering the low frequency of the TT genotype, the G-50T variant with TT and GT genotype was compared with the GG genotype. The G-50T variant was significantly more frequent in CAD patients (Table 2). To determine whether the association of the mutant genotypes with CAD frequency is independent of age, gender, and conventional cardiovascular risk factors, ORs (CAD with the G-50T
Discussion

We analyzed the CYP2J2 gene for genetic variants in patients with and without angiographically documented CAD. The frequency of coding SNPs was <1%. Functional importance of these relatively rare variants is not known. In contrast to the coding SNPs, the relatively frequent promoter polymorphism G-50T is much more likely to influence the cardiovascular risk of a population.

The G-50T variant has previously been described by King et al.11 with variable frequency in different racial groups. They also described 5 relatively rare (frequency <2%) coding region variants that previously were observed primarily in individuals of African descent. Thus, as a result of 2 independent sequencing studies of the entire CYP2J2 gene-coding region, there does not appear to be any frequent polymorphisms with amino acid substitutions in the CYP2J2 gene.

There are 4 putative Sp1 binding sites in the proximal CYP2J2 promoter, with their 5′-ends in positions −84, −72, −50, and −45. The loss of an Sp1 binding site in G-50T variants significantly reduces binding of Sp1. This finding is in accordance with our transfection studies demonstrating strong basal transcriotional activity from the WT but not G-50T CYP2J2 promoter, although no inducibility by cytokines and other substances able to induce CYP2C8/9 was observed. Constitutive expression of CYP2J2 mRNA and protein has been found in human heart tissue.1 These findings are consistent with the CYP2J2 promoter being TATA-less and that its basal activity is regulated predominantly by Sp1.11

<table>
<thead>
<tr>
<th>Wild Type (GG)</th>
<th>G-50T (GT, TT)</th>
<th>Difference in Covariables, OR (95% CI)</th>
<th>Adjusted Odds Ratio for CAD With G-50T (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=467</td>
<td>n=77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAD, %</td>
<td>51.2</td>
<td>64.9</td>
<td>1.77 (1.07–2.92)</td>
</tr>
<tr>
<td>Age, y</td>
<td>50.8±9.3</td>
<td>51.8±8.5</td>
<td>0.390</td>
</tr>
<tr>
<td>Male gender, %</td>
<td>76.6</td>
<td>70.1</td>
<td>0.216</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>27.9±4.1</td>
<td>27.5±4.4</td>
<td>0.422</td>
</tr>
<tr>
<td>Serum cholesterol, mg/dL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>211±48</td>
<td>216±52</td>
<td>0.483</td>
</tr>
<tr>
<td>LDL</td>
<td>136±42</td>
<td>142±50</td>
<td>0.353</td>
</tr>
<tr>
<td>HDL</td>
<td>40±15</td>
<td>43±17</td>
<td>0.228</td>
</tr>
<tr>
<td>Serum triglycerides, mg/dL</td>
<td>183±136</td>
<td>191±133</td>
<td>0.628</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>55.8</td>
<td>52.0</td>
<td>0.608</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>63.6</td>
<td>65.3</td>
<td>0.775</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>15.3</td>
<td>13.3</td>
<td>0.660</td>
</tr>
<tr>
<td>All covariables</td>
<td>...</td>
<td>...</td>
<td>2.23 (1.04–4.79)</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SD unless otherwise noted.
phism, and after adjustment for systemic hypertension, the association between G-50T SNP and CAD remains significant.

The incidence of adverse cardiovascular events is based on a retrospective analysis. Therefore, only nonfatal events were recorded. Additionally, a retrospective analysis is limited to what has been clinically documented. The incidence of acute coronary syndromes and cerebral ischemia needs further prospective evaluation, because a higher incidence of cardiovascular fatalities within 1 group could bias our findings. Nevertheless, our findings provide the first evidence for disease relevance of a polymorphism of a novel gene, CYP2J2. The association between the G-50T promoter polymorphism in younger patients and the prevalence of CAD further supports the role of P450-derived eicosanoids in limiting vascular inflammatory diseases such as atherosclerosis.

Acknowledgments

This work was supported by grants from the National Institutes of Health (HL-52233) to Dr Liao and from Alexander von Humboldt-Stiftung, FoRUM and Deutsche Forschungsgemeinschaft (DFG SP 537/3-1) to Dr Spiecker.

References

Risk of Coronary Artery Disease Associated With Polymorphism of the Cytochrome P450 Epoxygenase CYP2J2

Martin Spiecker, Harald Darius, Thomas Hankeln, Muhiidien Soufi, Alexander M. Sattler, Jürgen R. Schaefer, Koichi Node, Jan Börgel, Andreas Mügge, Klaus Lindpaintner, Anika Huesing, Bernhard Maisch, Darryl C. Zeldin and James K. Liao

Circulation. 2004;110:2132-2136; originally published online October 4, 2004;
doi: 10.1161/01.CIR.0000143832.91812.60

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/110/15/2132

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/