Association of PLA2G4A with myocardial infarction is modulated by dietary PUFAs$^{1–3}$

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ABSTRACT

Background: Leukotrienes are proinflammatory molecules derived from dietary PUFAs and have been associated with cardiovascular disease (CVD). We previously reported that an A→G variant (rs12746200) of the cytosolic phospholipase A2 group IVA gene (PLA2G4A), which encodes the enzyme that liberates PUFAs from cellular membranes for leukotriene synthesis, decreases the risk of CVD.

Objective: We sought to replicate these initial observations with a more clinically relevant phenotype, such as myocardial infarction (MI), and to determine whether dietary PUFAs mediate this association.

Design: In a Costa Rican case-control data set ($n = 3971$), rs12746200 was genotyped and was tested for an association with MI. Functional experiments were carried out to determine whether rs12746200 led to differences in mRNA expression.

Results: Risk of MI was significantly lower in AG/GG subjects than in AA homozygotes (OR: 0.86; 95% CI: 0.75, 0.99; $P = 0.040$). The reduced risk of MI was observed primarily in AG/GG subjects who were above the median for intake of dietary omega-6 ($n = 6$) PUFAs (OR: 0.71; 95% CI: 0.59, 0.87; $P$-interaction = 0.005). A similar analysis with dietary omega-3 ($n = 3$) PUFAs did not show a statistically significant nutrigenetic association ($P$-interaction = 0.23).

Conclusion: These results replicate the association of rs12746200 with CVD phenotypes and provide evidence that the protective association of this variant.

INTRODUCTION

Leukotrienes are mediators of inflammation synthesized from dietary PUFAs in various leukocytes, such as neutrophils and monocytes (1). Class 4 leukotrienes have potent proinflammatory properties and are derived from omega-6 ($n = 6$) PUFAs, such as arachidonic acid. By comparison, class 5 leukotrienes derived from omega-3 ($n = 3$) PUFAs, such as EPA and DHA, are much less biologically active. On activation of the cell by calcium, arachidonic acid or EPA is liberated from cellular membranes by the enzyme cytosolic PLA2G4A and oxidized by ALOX5 to form either LTA4 or LTA5, respectively. These intermediaries are subsequently converted to LTE4/LTE5 via enzymatic reactions by LTA4H; and LTC4S, respectively (1). Leukotrienes then affect the function of target cells, including monocytes and other proinflammatory leukocytes through receptor-mediated signal transduction.

Whereas leukotrienes have long been known to be involved in chronic allergic inflammatory conditions, such as asthma, the leukotriene pathway has also recently garnered attention for its potential role in CVD$^4$ (2–5). This stems from a series of recent biochemical, genetic, and pharmacologic studies in mice and humans that have provided evidence for the proatherogenic role of leukotrienes. For example, genetic deficiency of ALOX5 protects against aortic lesion formation and leads to other metabolic disturbances (6–9). Other mouse studies have implicated ALOX5 activating protein and the leukotriene receptors in the involvement of atherosclerosis-related traits as well (10–15). In humans, genetic variation in ALOX5, ALOX5 activating protein, LTA4H, and LTC4S has also been associated with various CVD phenotypes (16–24). Interestingly, promoter variants of ALOX5 have been shown to interact with dietary PUFAs to influence carotid atherosclerosis and risk of MI as well (16, 25). More recently, we carried out a comprehensive genetic evaluation of the leukotriene pathway, which replicated some of these previously reported associations, and provided evidence that a vari-

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$^4$ Abbreviations used: ABI, Applied Biosystems Inc; ALOX5, arachidonic acid 5-lipoxygenase; CVD, cardiovascular disease; FFQ, food-frequency questionnaire; HAE, human aortic endothelial cell; LT, leukotriene; LTA4H, leukotriene A4 hydrolase; LTC4S, leukotriene C4 synthase; MI, myocardial infarction; ox-PAPC, oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphatidylcholine; PLA2G4A, phospholipase A2 group 4A.

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iant of PLA2G4A (rs12746200) was associated with a decreased risk of CVD (26). In the current study, we sought to replicate the association of PLA2G4A with a more clinically significant CVD phenotype, namely MI, and determine whether the association could be mediated by dietary PUFAs.

SUBJECTS AND METHODS

Subjects

Cases for this study were adult patients who were survivors of a first acute MI, as diagnosed by a cardiologist at any of the recruiting hospitals in the Central Valley of Costa Rica. A study cardiologist confirmed all cases according to the WHO criteria for MI, which requires typical symptoms plus either elevation in cardiac enzyme concentration or diagnostic changes in the electrocardiogram. Enrollment was carried out in the step-down unit of the recruiting hospitals, and cases were ineligible for participation if they 1) died during hospitalization, 2) were older than 75 y on the day of their first MI, 3) were physically or mentally unable to answer the questionnaire, or 4) had a previous hospital admission related to CVD. For each case, one population-based control subject, matched for age (± 5 y), sex, and county of residence, was recruited. The controls were randomly selected by using data from the National Census and Statistics Bureau of Costa Rica. Control subjects were ineligible if they had ever had an MI or if they were physically or mentally unable to answer the questionnaires. The catchment area consisted of 34 counties in the Central Valley of Costa Rica, and the participants were recruited between 1994 and 2004. Participation was 98% for cases and 88% for controls. All subjects gave informed consent on documents approved by the Human Subjects Committee of the Harvard School of Public Health and University of Costa Rica. Approval for the current study was also obtained from the Institutional Review Board of the University of Southern California Keck School of Medicine.

Trained personnel visited all study participants at their homes for data collection. Fieldworkers collected anthropometric measurements while the study subjects were wearing light clothing and no shoes. All measurements were performed in duplicate, and the average was used for analyses. Sociodemographic characteristics, medical history, and lifestyle habits were collected by using a general questionnaire. Dietary intake data were collected by using an FFQ that has been developed and validated specifically to assess fatty acid intake among the Costa Rican population (27). The validity coefficient for the assessment of arachidonic acid with the use of the FFQ was high (0.53)—a finding consistent with the performance of the FFQ for other fatty acids (27)—and the correlation coefficient between the FFQ and seven 24-h recalls was 0.62. Biological samples were always collected in the morning after an overnight fast. Blood samples (20 mL) were drawn into 0.1% EDTA-containing tubes after a 12–14 h fast and were immediately stored at 4°C. Within 36 h, the samples were centrifuged at 2500 rpm for 20 min at 4°C to isolate and to separate plasma into aliquots. The samples were then sealed and stored under nitrogen at −80°C until analysis.

For the current analyses, genotype information, complete data on all the descriptive variables, and potential confounders were available from 1936 cases and 2035 controls.

Genotyping

Genotyping of the PLA2G4A single nucleotide polymorphism rs12746200 was carried out with the ABI TaqMan system (28) by using a custom assay from ABI’s “Assays by Design” service.

Gene-expression analyses

HAECs were isolated from aortic explants of 132 heart transplant donors, as described previously (29). Briefly, the extracted cells were grown to 90% confluence and treated in duplicate for 4 h either with control media or with media containing ox-PAPC. RNeasy and DNeasy kits (Qiagen Inc) were used to isolate cytosolic RNA and genomic DNA. PLA2G4A mRNA levels were determined by using Affymetrix HT-HU133A microarrays, as described elsewhere (29). Intensity values were normalized with the RMA normalization method in R 2.5.0 by using justRMA function in the Bioconductor affy package. Expression values were averaged between duplicate arrays per treatment and study subject (29). Genotyping was performed by the ABI TaqMan system as described above.

Statistical analyses

The significance of differences in health characteristics and potential confounders were assessed by Wilcoxon’s rank-sum test for continuous variables and by chi-square test for binary variables. Before all analyses, rs12746200 was tested for Hardy-Weinberg equilibrium by using a chi-square test. ORs and 95% CIs were estimated from multiple unconditional logistic regression assuming a dominant genetic model. Median intake of dietary omega-6 (6.93 g/d) and omega-3 (1.02 g/d) PUFAs in the control group were used to stratify subjects into low and high groups. The fully adjusted model included age, sex, county of residence, percentage of total energy from fat, smoking, household income, history of diabetes, hypertension, or hypercholesterolemia, obesity, and family history of MI. Because of the high correlation in the intake of dietary PUFAs, the gene-dietary interaction analyses were additionally adjusted for the reciprocal PUFA, whereby the amounts of dietary omega-6 and omega-3 were treated as continuous variables. Other variables tested, but not included in the final model, were total calorie intake, saturated fat intake, monounsaturated fat intake, polyunsaturated fat intake, cholesterol intake, fiber intake, and ethnicity. Differences in the mean gene expression levels in HAECs were evaluated by using a linear regression. Expression levels were normally distributed both for untreated and ox-PAPC–treated HAECs. The donors of the HAECs were anonymous; thus, individual information, such as ethnicity, was unknown. As described by Romanoski et al (29), modest population stratification was seen by using principal component analysis for these data, although removing samples outside the 2 first PCs did not affect the results of their study. All data were analyzed with the Statistical Analysis Systems software version 9.2 (SAS Institute Inc) or with the Stata version 8.2 (StataCorp LP).

RESULTS

Clinical characteristics of the study population

The clinical characteristics of the Costa Rican cases and controls used for this study are shown in Table 1. As expected,
the cases exhibited several risk factors associated with MI, including being more likely to have diabetes, hypertension, a family history of MI, lower household income, and consumption of more calories per day, including a higher intake of dietary cholesterol and various fatty acids (Table 1).

Association of PLA2G4A with MI

To replicate our previously reported association of PLA2G4A, we genotyped the rs12746200 A→G polymorphism in 3971 individuals from the Costa Rican data set. The frequency of the G allele was ~14% in Costa Ricans and was comparable with that observed in subjects of Northern European descent (26). To increase the power for detecting an association, we assumed a dominant genetic model in our analyses. As shown in Table 2, the risk of MI was significantly lower in AG/GG individuals homozygous for the A allele (OR: 0.86; 95% CI: 0.75, 0.99; P = 0.04). Adjustment for covariates, such as age, sex, area of residence, total fat intake, smoking, and household income, did not attenuate this association (Table 2). Inclusion of history of diabetes, hypertension, or hypercholesterolemia; obesity; and family history of MI as additional covariates did not change the effect estimate for the association of rs12746200 with MI but decreased the statistical significance, which may have been due, in part, to a reduced sample size in the fully adjusted model (Table 2). Using a panel of ancestry informative markers that were available in this data set (31), we also included the proportion of European, West African, and Amerindian admixture as covariates in these analyses. However, this adjustment also did not alter the results (data not shown).

Nutrigenetic interaction between PLA2G4A and dietary PUFAs on risk of MI

We next determined whether the association of rs12746200 with MI was modulated by dietary amounts of omega-6 or omega-3 PUFAs. Given that this variant decreases the risk of MI, we used AA homozygotes with above-median intakes of dietary omega-6 PUFAs (>6.93 g/d) as the reference group because these subjects would be considered at most risk of MI (Table 3). Although no association was observed in the low dietary omega-6 PUFA group, these analyses showed a significant gene-dietary interaction, whereby a decreased risk of MI was observed in AG/GG subjects with above-median dietary omega-6 PUFAs (OR: 0.71; 95% CI: 0.59, 0.87; P-interaction = 0.005). These results remained significant even after the addition of various covariates in the model (Table 3). Gene-dietary interaction analyses using tertiles of omega-3 PUFA intake also yielded consistent results (see Supplementary Table 1 under “Supplemental data” in the online issue). We next carried out the reciprocal analysis with dietary omega-3 PUFAs with AA homozygotes with below-median dietary omega-3 intakes (<1.02 g/d) as the reference group (Table 4). This analysis did not show a statistically significant interaction between genotype, dietary omega-3 PUFAs, and risk of MI (Table 4). An analysis using tertiles of omega-3 PUFA intake also showed no significant gene-dietary interaction.

TABLE 1
General characteristics of the study population by case-control status

<table>
<thead>
<tr>
<th>Trait</th>
<th>Controls (n = 2035)</th>
<th>Cases (n = 1936)</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (%)</td>
<td></td>
<td></td>
<td>0.87</td>
</tr>
<tr>
<td>Male</td>
<td>74</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>26</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>58 ± 11</td>
<td>58 ± 11</td>
<td>0.76</td>
</tr>
<tr>
<td>History of hypercholesterolemia (%)</td>
<td>26.9</td>
<td>30.3</td>
<td>0.02</td>
</tr>
<tr>
<td>History of diabetes (%)</td>
<td>14.2</td>
<td>24.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>History of hypertension (%)</td>
<td>29.5</td>
<td>38.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Family history of MI (%)</td>
<td>7.8</td>
<td>12.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BMI &gt;30 (%)</td>
<td>17.2</td>
<td>13.7</td>
<td>0.003</td>
</tr>
<tr>
<td>Current smokers (%)</td>
<td>21.9</td>
<td>40.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ever smokers (%)</td>
<td>60.7</td>
<td>69.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Monthly household income (US$)</td>
<td>569 ± 423</td>
<td>500 ± 390</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>European admixture (%)</td>
<td>57.8 ± 7.9</td>
<td>57.5 ± 8.1</td>
<td>0.20</td>
</tr>
<tr>
<td>Amerindian admixture (%)</td>
<td>38.4 ± 7.4</td>
<td>38.5 ± 7.4</td>
<td>0.60</td>
</tr>
<tr>
<td>West African admixture (%)</td>
<td>3.9 ± 3.5</td>
<td>4.1 ± 4.1</td>
<td>0.20</td>
</tr>
<tr>
<td>Total energy (kcal/d)</td>
<td>2453 ± 773</td>
<td>2709 ± 951</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Dietary cholesterol (mg/1000 kcal)</td>
<td>117.9 ± 52.4</td>
<td>127.0 ± 58.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Dietary fiber (g/d)</td>
<td>8.2 ± 3.3</td>
<td>8.5 ± 3.3</td>
<td>0.0005</td>
</tr>
<tr>
<td>Total fat (% of total energy)</td>
<td>31.8 ± 5.8</td>
<td>32.4 ± 5.9</td>
<td>0.002</td>
</tr>
<tr>
<td>Saturated fat (% of total energy)</td>
<td>10.4 ± 2.7</td>
<td>11.1 ± 2.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Monounsaturated fat (% of total energy)</td>
<td>11.8 ± 3.8</td>
<td>11.9 ± 3.5</td>
<td>0.007</td>
</tr>
<tr>
<td>Omega-6 (n−6) PUFAs (g/d)</td>
<td>9.4 ± 7.1</td>
<td>10.2 ± 8.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Omega-3 (n−3) PUFAs (g/d)</td>
<td>1.13 ± 0.57</td>
<td>1.20 ± 0.69</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*Two-sided P values are reported between cases and controls (derived from Wilcoxon’s rank-sum test for continuous variables, or a chi-square test for dichotomous variables). MI, myocardial infarction.

* Mean ± SD (all such values).

† Adjusted for total energy intake by using the residuals method (30).
Functional characterization of rs12746200

To determine whether rs12746200 is a functional variant, we evaluated \(PLA2G4A\) gene expression levels in HAECs as a function of genotype. HAECs from 132 donors were treated for 4 h with either media alone or ox-PAPC. The expression of \(PLA2G4A\) was normally distributed under both treatment conditions and did not change in response to treatment with ox-PAPC (data not shown). As shown in Figure 1, gene expression levels under control conditions were marginally, but significantly, lower (\(P = 0.014\)) in AG/GG subjects (8.6 ± 0.13) than in AA homozygotes (8.9 ± 0.06). \(PLA2G4A\) mRNA levels were similarly lower (\(P = 0.012\)) in AG/GG subjects (8.7 ± 0.14) than in AA homozygotes (9.1 ± 0.06) when the cells were treated with ox-PAPC (Figure 1).

### DISCUSSION

In the current study, we provide evidence that the risk of MI was modestly lower (~15%) in AG/GG carriers of the \(PLA2G4A\) rs12746200 variant than in AA subjects. This association remained significant in a fully adjusted model that included admixture proportions as covariates and was supported by the relatively weak, but significant, effect of rs12746200 on \(PLA2G4A\) expression levels (discussed further below). These results are consistent with our recent observations that rs12746200 was associated with a decreased risk of CVD in...
a white patient population of Northern European descent (26). However, rs12746200 had a stronger association in whites, in whom it reduced both the risk of prevalent CVD and future major adverse cardiac events by 30% despite being slightly less frequent (10%). Such differences are not entirely surprising in genetic association studies and can be attributed to various factors, including those related to the study populations, sample size, functional effects, and/or the CVD phenotypes being investigated. Nonetheless, these results replicate our previous observations with a more clinically significant CVD outcome and in an independent case-control data set of different ethnicity.

Another important aspect of our study is the nutrigenetic analyses, which demonstrate that the cardioprotective association of rs12746200 occurs primarily in AG/GG subjects who had high dietary omega-6 PUFA intakes. These results suggest that rs12746200 can mitigate, in part, the pro-atherogenic effects of omega-6 PUFAs in this Costa Rican population (25). However, the G allele does not appear to provide any further risk reduction in subjects who have low omega-6 PUFA intakes. In addition, omega-6 PUFAs are direct substrates for PLA2G4A, which supports the notion that observed gene-dietary interactions reflect true biological associations. Of note, these analyses were adjusted for levels of the reciprocal group of dietary omega-3 PUFAs, which, by comparison, did not show significant interactions with genotype on risk of MI. This may be due to the relatively low consumption of fish by the population in the Central Valley region of Costa Rica (32), from which this sample was collected. Taken together with previous studies (16, 25), these results suggest that CVD phenotypes in humans can be influenced through interactions between genetic variation in the leukotriene pathway and dietary PUFAs that serve as substrates for the biosynthetic enzymes. It would be of interest to determine whether other leukotriene pathway genes that have been associated with CVD (17, 18, 20–22) exhibit nutrigenetic interactions as well.

PLA2G4A encodes a calcium-dependent cytosolic phospholipase that is expressed in a variety of tissues, including endothelial cells (33). In addition to liberating PUFAs from cellular membranes for synthesis of lipid mediators, PLA2G4A also upregulates endothelial cell expression of intracellular adhesion molecule 1 (34), which is a known mediator of monocyte recruitment to the artery wall. In this regard, our functional experiments are consistent with rs12746200 being associated with a decreased risk of MI because PLA2G4A mRNA levels were lower in HAECS from AG/GG subjects than from AA homozygotes. This would presumably lead to lower levels of...

**TABLE 4**

Interaction between PLA2G4A and dietary omega-3 (n=3) PUFAs on risk of MI

<table>
<thead>
<tr>
<th>Model and genotype group</th>
<th>Low dietary omega-3 PUFAs (&lt;1.02 g/d)</th>
<th>High dietary omega-3 PUFAs (≥1.02 g/d)</th>
<th>P-interaction*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>OR (95% CI)</td>
<td>n</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>1400</td>
<td>1.00</td>
<td>1500</td>
</tr>
<tr>
<td>AG/GG</td>
<td>518</td>
<td>0.95 (0.77, 1.16)</td>
<td>543</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>1400</td>
<td>1.00</td>
<td>1500</td>
</tr>
<tr>
<td>AG/GG</td>
<td>518</td>
<td>0.95 (0.77, 1.16)</td>
<td>543</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>1277</td>
<td>1.00</td>
<td>1403</td>
</tr>
<tr>
<td>AG/GG</td>
<td>473</td>
<td>0.93 (0.75, 1.16)</td>
<td>508</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>1183</td>
<td>1.00</td>
<td>1329</td>
</tr>
<tr>
<td>AG/GG</td>
<td>436</td>
<td>0.97 (0.77, 1.22)</td>
<td>488</td>
</tr>
</tbody>
</table>

* Data are reported as ORs with 95% CIs, as estimated by using unconditional logistic regression analysis. The reference genotype group for each model is AA in the high dietary omega-6 PUFA strata. MI, myocardial infarction.

* Model 1 was adjusted for age, sex, area of residence, and omega-6 PUFAs. Model 2 was adjusted as for model 1 plus percentage of total energy intake and fat intake. Model 3 was adjusted as for model 2 plus smoking and household income. Model 4 was adjusted as for model 3 plus history of diabetes, hypertension, or hypercholesterolemia; obesity; and family history of MI.

* P values were derived from Wald’s chi-square test.

**FIGURE 1.** Mean (±SEM) endothelial cell gene-expression levels as a function of rs12746200 genotype. Under control conditions (untreated) or after incubation with ox-PAPC, PLA2G4A mRNA levels were significantly lower in human aortic endothelial cells from AG/GG subjects than in AA homozygotes. PLA2G4A mRNA levels were obtained from Affymetrix HT-HU133A microarrays, and a t test was used to assess the difference in means between 2 genotype groups. *P = 0.01. ox-PAPC, oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphatidylcholine.
poinflammatory class 4 leukotrienes (and possibly intracellular adhesion molecule 1) and thus provide a biologically plausible mechanism for why rs12746200 decreases the risk of MI primarily in the context of high dietary omega-6 PUFAs. However, we are not able to definitely prove this hypothesis because leukotriene production was not determined in the HAECC samples used herein. Moreover, other classes of arachidonic acid and EPA-derived lipid mediators, such as lipoxins, resolvins, and maresins, have been recognized as having important roles in the resolution of inflammatory processes (35). Thus, determining whether these PUFA derivatives are different as a function of PLA2G4A genotype may also provide further insight into the molecular mechanism by which rs12746200 leads to a decreased risk of MI. Last, rs12746200 is located in intron 3 of PLA2G4A and it is not known whether this region contains a regulatory element that controls gene expression. On the basis of HapMap data for Hispanics, rs12746200 is not in linkage disequilibrium with other variants of PLA2G4A, including a functional K651R polymorphism (rs2307198) that increases in vitro activity by nearly 2-fold (36). Thus, additional studies will be required to determine whether rs12746200 is the causal variant underlying the association we observe with MI or is linked to another heretofore unidentified single nucleotide polymorphism that affects expression levels and/or enzyme activity.

This study had several limitations that should be considered. First, MI was defined according to WHO criteria based on clinical symptoms, electrocardiogram abnormalities, and elevated cardiac enzymes. By comparison, the universal definition of MI uses more sensitive and specific serologic biomarkers and more specific imaging techniques (37). Thus, there is the potential for disease misclassification in our study, although this was unlikely to be different with regard to PLA2G4A genotype and would only bias the effect estimates toward the null. Additionally, because this study sample consisted of Costa Ricans, the nutrigenetic bias the effect estimates toward the null. Additionally, because

REFERENCES


