Vitamin C and E Intake Is Associated With Increased Paraoxonase Activity

Gail P. Jarvik, Nancey Trevanian Tsai, Laura A. McKinstry, Roohi Wani, Victoria H. Brophy, Rebecca J. Richter, Gerard D. Schellenberg, Patrick J. Heagerty, Thomas S. Hatuskami, Clement E. Furlong

Objective—Paraoxonase (PON1), an esterase physically associated with high density lipoprotein, has been shown to inhibit atherogenic low density lipoprotein and high density lipoprotein oxidation. PON1 activity appears to be primarily under genetic control with some environmental modification and is a predictor of vascular disease. Vitamins C and E, dietary antioxidants, scavenge free-oxygen radical products that may depress PON1 activity. Therefore, we evaluated the relationship between dietary vitamin C and E intake and PON1 activity.

Methods and Results—The vitamin C and E intakes of male white subjects (n=189) were estimated by using a standardized food frequency survey. With covariates, vitamin C or E intakes were found to be significant positive predictors of PON1 activity for the hydrolysis of paraoxon and diazoxon with the use of linear regression. Smoking and use of statins were independent predictors of PON1 activity.

Conclusions—PON1 activity, which is primarily genotype dependent, varies with antioxidant vitamins, cigarette smoking, and statin drug use. Because PON1 activity is a better predictor of vascular disease than is the currently described genetic variation in PON1, further studies of the environmental influences on PON1 activity and additional PON1 genetic variants are warranted. (Arterioscler Thromb Vasc Biol. 2002;22:189–197.)

Key Words: cardiovascular • antioxidants • genetics • diet • vitamins • paraoxonase • smoking

Paraoxonase (PON1) genotype and activity have been implicated in the pathogenesis of atherosclerosis. This calcium-dependent esterase is physically associated with HDL. Paraoxonase may protect against vascular disease through its inhibition of LDL oxidation. Studies have demonstrated that PON1 activity is lower in patients with carotid artery disease, coronary heart disease, and myocardial infarction, even when no marginal effect of PON1 genotype on disease is notable.

The activity of PON1 is under genetic and environmental regulation. The PON1L55M polymorphism, which results in Arg instead of Gln at amino acid position 192, has been shown to account for 73% to 76% of the variation in hydrolysis activity of PON1 for paraoxon (POase activity) and 12% to 25% of the variation in activity for the hydrolysis of diazoxon (DZOase activity) in vitro with the use of an assay in the presence of 2 mol/L NaCl. The PON1L55M polymorphism seems to be correlated with the PON1 protein concentration through linkage disequilibrium with promoter polymorphisms and through increased protein turnover. Of the 5' regulatory promoter region polymorphisms, PON1C-108T appears to have the largest effect on PON1 concentration, accounting for 23% of the variance in PON1 arylesterase activity.

Environmental factors that alter PON1 activity include tobacco consumption, which has been reported to depress PON1 activity and concentration. PON1 POase activity has been found to be increased by pharmacological therapy with simvastatin and hormone replacement therapy. Fatty meals may depress postprandial PON1 arylesterase activity. The concentration of PON1 mRNA has been shown to increase or decrease when mice are challenged with an atherogenic diet in a strain-dependent fashion. Pomegranate juice has been reported to increase PON1 activity in mice. Exposure to organophosphate pesticides does not induce PON1 activity.

The relationship between antioxidant intake and atherosclerosis continues to be studied, with many conflicting results. These studies have been reviewed by Berliner and Tribble, among others. Antioxidant supplements may be expected to influence PON1 activity by altering oxidative stress. The goal of the present study was to examine the effect of the dietary antioxidants vitamin C (ascorbic acid) and vitamin E (eg, α-tocopherol) on PON1 activities measured by the hydrolysis of paraoxon and diazoxon to determine whether the role of the vitamins and PON1 antioxidant effects might be interrelated.

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Methods

Subjects
The subjects were white male veterans. The mean age was 65 years, with a range of 48 to 88 years. There were 189 subjects: 94 men with <15% carotid stenosis bilaterally, 23 men with 16% to 49% carotid stenosis, 21 men with 50% to 79% carotid stenosis, 31 men with >80% carotid stenosis and/or with carotid endarterectomy, and 20 men with lower extremity vascular disease without carotid stenosis. Exclusionary criteria included familial hypercholesterolemia, total fasting cholesterol >400 mg/dL, hypercoagulable state, and/or use of anticoagulant medication or inability to consent. Human Subjects Oversight Committees at the University of Washington Medical Center and the Veterans Affairs Puget Sound Health Care centers approved the study, and written informed consent was obtained from all participants.

Survey Methods
Subjects were asked to complete a standardized food frequency survey from The Health Professionals Follow-Up Study (1988, Brigham and Women’s Hospital). The survey asked about (1) the average frequency of intake over the previous year of specified portions of 131 foods and (2) the use of vitamins and mineral supplements, including dose and duration of multivitamins, vitamin C, and vitamin E. Questions regarding brand of multivitamins and breakfast cereals used were asked to clarify the quantities of specific vitamin supplementation. Excluded from the analysis were those subjects (1) whose caloric intake was not between 800 and 4200 kcal/d and (2) whose surveys had >70 blank items of a total of 131 items. All vitamin usage was energy-adjusted to 2000 kcal/d. This food frequency survey has been validated against two 1-week diet records taken ~6 months apart.9 In that study, the adjusted correlation coefficients between the food frequency questionnaire and both 1-week diet records, which were adjusted for energy intake and within-person variability in daily intake, were 0.92 for vitamins C and E. The survey has predicted cardiovascular disease in relation to fat intake,80 ω-3 fatty acid intake,81 fiber,82,83 and vitamin E consumption.82

Smoking status was ascertained by survey. Ten subjects who reported that they did not currently smoke but reported 0 months since the last cigarette were coded as current smokers. This code was given because it seems likely that these subjects are current smokers with the intention of quitting and because a prior study suggested that the effects of smoking on PON1 remain for months after tobacco cessation.83 The use of statins and other medications was ascertained from Veterans Affairs Puget Sound Health Care pharmacy records.

PON1 Genotype and Activity Phenotype Methods
DNA was prepared from buffy coat preparations by a modification of the procedure of Miller and Dykes,86 with the use of Puregene reagents (Gentra). PON1192 genotypes were determined by using polymerase chain reaction techniques and AlwI restriction enzyme analysis.87 The PON1108 promoter region polymorphism was typed as recently described.12

PON1 paraoxon (POase activity) and diazoxon (DZOase activity) hydrolysis rates were measured spectrophotometrically with liver heparin plasma, as previously described.88 All samples were run in duplicate; the averaged value was used for analysis. PON1192 genotype can be predicted with high accuracy from examination of the 2D plot of paraoxon and diazoxon hydrolysis rates.5,88 When assignments did not match, genotyping and phenotyping studies were repeated. One hundred eighty (95.2%) of the 189 subjects had PON1192 genotype-phenotype agreement. These mismatches are likely due to other PON1 variants that influence PON1 activity.12 Thus, the PON1 activity assays provide functional genomics for variants that are not typed for and also reflect environmental influences.

Lipid and HbA1c Measurements
Lipid measurements were performed on fasting whole plasma. Standard enzymatic methods were used to determine the levels of total cholesterol, triglycerides, and HDL cholesterol (HDL-C) on an Abbott Spectrum analyzer.39–41 LDL cholesterol was calculated.42 Hemoglobin A1c (HbA1c) measurement used standard high-pressure liquid chromatography.43 This assay does separate pre-HbA1c from HbA1c, eliminating the false elevation that can result from high serum glucose if pre-HbA1c is not removed.

Statistical Analysis
Marginal associations between vitamin intake and smoking status or statin use were investigated by ANOVA. Linear regression was used to determine whether vitamin C or vitamin E intake predicted PON1 POase and DZOase activities. The subjects’ ages and dummy variables for their PON1192 and PON1108 genotype, whether or not they currently smoked, and pharmacological statin therapy for lipid lowering were considered as covariates. PON1192QQ and PON1108QO genotypes were used as the reference genotype (coded 0,0) for the dummy variable regression. All 4 quantitative variables significantly deviated from a normal distribution. A natural logarithmic transformation was required for the vitamin C (ln-vitC) intake variable. A square root transformation was used to adjust the vitamin E intake (sq-vitE), DZOase (sq-DZOase), and POase (sq-POase) variables. When total calories of dietary fat and grams of alcohol consumed were considered as covariates, neither predicted PON1 activities, and neither changed the relationship of vitamin C or E with PON1 activities (results not shown). HDL-C level was used as an additional covariate. Multiplicative interaction for the prediction of PON1 activity between PON1192 and PON1108 genotype dummy variables and the antioxidant intakes were not significant and are not reported. Power to detect interactions was expected to be low. The residuals of the regression of age, current smoking, statin use, and PON1192 and PON1108 genotypes on sq-PON1 activity were relocated to the activity means and resquared; they are described for each vitamin intake tertile. Because of high correlations between the antioxidant intakes and between the PON1 activities, adjustments for multiple contrasts were not made. All analyses used SPSS for Windows (version 10.0.5).

Results
As seen in Table 1, the correlation of ln-vitC with sq-vitE was 0.58 (P=0.01). sq-POase and sq-DZOase are highly correlated within PON1192 genotypes (0.77, 0.56, and 0.84 for the PON1192 QQ, QQ, and RR genotypes, respectively (all P<0.001). There was a trend toward lower ln-vitC (P=0.06) intake in current smokers. Decreased sq-vitE (P=0.25) intake in smokers was not significant. The ln-vitC (P=0.9) and sq-vitE (P=0.4) intakes did not differ significantly between users and nonusers of statins.

Dietary intakes of vitamin C and of vitamin E were significant predictors of PON1 activity with the use of POase (P=0.011 and 0.005, respectively) and DZOase (P=0.001 for vitamins C and E) activities as measures for linear regression with covariates (Table 2). Increased vitamin C and E intakes were associated with an elevation of PON1 activities. Inclusion of either ln-vitC or sq-vitE into a regression that included the other did not result in a significant improvement in sq-POase or sq-DZOase prediction. Although normalization is required for appropriate statistical testing, we also computed the regression coefficients without normalizing transformations of PON1 activities or vitamin intake. The multiple regression coefficients for vitC (milligrams per day) and vitE (IU per day) prediction of POase activity (units per
liter) were 0.08 and 0.13, respectively. For the prediction of DZOase activity, these were 1.72 and 1.81, respectively.

When all covariates were included in the regression, including either vitamin C or E as predictors, PON1 POase and DZOase activities declined with age (all \( P < 0.009 \)). Current smoking predicted depressed POase activity (all \( P < 0.04 \)) but was not predictive of DZOase activity (all \( P > 0.7 \)). When total calories of dietary fat and grams of alcohol consumed were considered as covariates, neither predicted PON1 activities, and neither changed the relationship of vitamin C or E with PON1 activities (results not shown). HDL-C level was uncorrelated with the vitamin intakes but was a significant predictor of sq-POase and sq-DZOase when added to the regression model containing age, smoking, statin use, \( PON1_{108} \) and \( PON1_{192} \) genotype, and either vitamin C or E intake (all \( P < 0.013 \)). The effects of vitamin C or E intake on POase and DZOase activity remained significant when HDL-C was added to the regression model (all \( P < 0.012 \)).

When sq-POase was adjusted for age and \( PON1_{108} \) and \( PON1_{192} \) genotype effects with the use of linear regression, 10.7% of the remaining POase variance was attributable to sq-vitE, smoking status, and statin use, or 10.4% was from ln-vitC, smoking status, and statin use. The marginal effect

### TABLE 1. Subject Characteristics

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>189</td>
</tr>
<tr>
<td>Men, %</td>
<td>100</td>
</tr>
<tr>
<td>White, %</td>
<td>100</td>
</tr>
<tr>
<td>Taking hypoglycemics, %</td>
<td>26.5</td>
</tr>
<tr>
<td>Taking lipid-lowering medication, %</td>
<td>33.3</td>
</tr>
<tr>
<td>Statin drug use, %</td>
<td>19.0</td>
</tr>
<tr>
<td>Taking antihypertensives, %</td>
<td>66.1</td>
</tr>
<tr>
<td>Current smokers, %</td>
<td>29.6</td>
</tr>
<tr>
<td>Pack years smoked, mean y</td>
<td>29.1</td>
</tr>
<tr>
<td>Total cholesterol, mean mg/dL</td>
<td>195.3</td>
</tr>
<tr>
<td>Calculated LDL-C, mean mg/dL</td>
<td>110.5</td>
</tr>
<tr>
<td>Triglycerides, mean mg/dL</td>
<td>183.9</td>
</tr>
<tr>
<td>VLDL-C, mean mg/dL</td>
<td>35.7</td>
</tr>
<tr>
<td>HDL-C, mean mg/dL</td>
<td>42.0</td>
</tr>
<tr>
<td>Poase, U/L (sqrt-POase)</td>
<td>566.4 (23.8)</td>
</tr>
<tr>
<td>DZOase, U/L (sqrt-DZOase)</td>
<td>9920.2 (99.6)</td>
</tr>
</tbody>
</table>

### TABLE 2. Dietary Antioxidant Effects on PON1 Activities

<table>
<thead>
<tr>
<th>Predictor</th>
<th>B for sq-POase (SE)</th>
<th>( P )</th>
<th>B for sq-DZOase (SE)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Considering vitamin C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In-vitamin C</td>
<td>0.54 (0.21)</td>
<td>0.011*</td>
<td>2.78 (0.74)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Current smokers</td>
<td>-1.15 (0.55)</td>
<td>0.039*</td>
<td>0.63 (1.93)</td>
<td>0.744</td>
</tr>
<tr>
<td>( PON1_{108Q} ) genotype</td>
<td>12.40 (0.52)</td>
<td>&lt;0.0001*</td>
<td>-13.98 (1.81)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>( PON1_{108P} ) genotype</td>
<td>22.15 (1.00)</td>
<td>&lt;0.0001*</td>
<td>-30.58 (3.52)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>( PON1_{192Q} ) genotype</td>
<td>4.80 (0.71)</td>
<td>&lt;0.0001*</td>
<td>22.14 (2.49)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>( PON1_{192R} ) genotype</td>
<td>3.08 (0.60)</td>
<td>&lt;0.0001*</td>
<td>12.17 (2.12)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Statin drug use</td>
<td>1.55 (0.62)</td>
<td>0.014*</td>
<td>2.98 (2.19)</td>
<td>0.174</td>
</tr>
<tr>
<td>Age</td>
<td>-0.10 (0.03)</td>
<td>&lt;0.0001*</td>
<td>-0.25 (0.09)</td>
<td>0.009*</td>
</tr>
</tbody>
</table>

| Considering vitamin E                  |                     |        |                      |        |
| sq-vitamin E                           | 0.08 (0.03)         | 0.005* | 0.32 (0.10)          | 0.001*   |
| Current smokers                        | -1.21 (0.55)        | 0.028* | 0.17 (1.94)          | 0.931   |
| \( PON1_{108Q} \) genotype            | 12.35 (0.51)        | <0.0001* | -14.24 (1.83)       | <0.0001* |
| \( PON1_{108P} \) genotype            | 22.04 (0.99)        | <0.0001* | -31.39 (3.53)       | <0.0001* |
| \( PON1_{192Q} \) genotype            | 4.80 (0.71)         | <0.0001* | 22.23 (2.51)        | <0.0001* |
| \( PON1_{192R} \) genotype            | 3.07 (0.60)         | <0.0001* | 12.30 (2.14)        | <0.0001* |
| Statin drug use                        | 1.67 (0.62)         | 0.008* | 3.49 (2.21)          | 0.116   |
| Age                                    | -0.10 (0.03)        | <0.0001* | -0.26 (0.09)        | 0.008*   |

*\( P < 0.05 \); regressions include either ln-vitamin C or sq-vitamin E. \( PON1_{108Q} \) and \( PON1_{192Q} \) are reference genotypes. See methods.
attributable to either ln-vitC or sq-vitE was 3.6% or 4.0%, respectively. The marginal effects of smoking and statins use were 3.5% and 4.0%, respectively. When sq-DZOase was adjusted for age and PON1 192 genotype effects, 7.8% of the remaining POase variance was attributable to sq-vitE, smoking status, and statin use, or 10.2% was from ln-vitC, smoking status, and statin use. The marginal effect attributable to either ln-vitC or sq-vitE was 8.1% or 5.2%, respectively. The marginal effects of smoking and statin use were 0% and 2.2%, respectively. The portion of variance attributable to these environments will vary with the prevalence of each factor. The mean POase activity, adjusted for age, statin use, and PON1 192 genotype was 549.4 U/L for the lowest vitamin C intake tertile and 602.2 U/L for the highest vitamin C intake tertile. The adjusted POase activity means for the lowest and highest vitamin E intake tertiles were 549.0 and 602.2 U/L, respectively. The mean adjusted DZOase activities for the lowest and highest vitamin E intake tertiles were 9298.7 and 10675.0 U/L, respectively; for vitamin E, they were 9482.9 and 10453.0, respectively.

Discussion

In the present study, increased activity of PON1 (POase and DZOase) was predicted by increased vitamin C and E intake. The inhibition of LDL oxidation by HDL, which is due to the hydrolysis of lipid peroxidases and the resulting inhibition of lipid peroxides, appears to be, at least in part,6,44 – 46 a function of the enzyme paraoxonase, which is a component of HDL.47 Watson et al45 showed that PON1 destroyed the muti-oxygenated molecules found in oxidized phosphatidylcholine. Furthermore, they showed that inactivation of PON1 reduces the ability of HDL to inhibit LDL modification and also reduces the ability of HDL to inhibit monocyte-endothelial interactions, both of which appear to be important in the inflammatory response in arterial wall cells that promotes atherogenesis. PON1 reduced mildly oxidized phospholipids by eliminating oxidized derivatives of unsaturated fatty acids.6,45 Inactivation of PON1 by oxidized LDL can be inhibited by antioxidants.48

The role of antioxidant vitamins C and E in the prevention of vascular disease is still being clarified.27 Vitamin E may inhibit vascular lesion progression.49 Although the effects of PON1 on LDL oxidation appear to be independent of the function of antioxidant vitamins,45 vitamins C and E have been shown to inhibit LDL oxidation.50,51 Any reduction in oxidative stress related to vitamin C and E intake may preserve PON1 activity. The HDL effect on the protection of LDL from oxidation, mainly attributable to PON1, is more prolonged than the effects of antioxidant vitamins.52

Previous reports of PON1 depression by tobacco smoke in ex vivo assays53 are consistent with the lower POase activity associated with current smoking in the present study and a prior study.59 In a Costa Rican sample, the PON1 192 genotype was associated with myocardial infarction only in nonsmokers,54 suggesting the importance of jointly considering environmental factors that modify PON1 activity. Additionally, elevation of PON1 activity in statin drug users was consistent with the reported effects of simvastatin on PON1 activity.16

The present study has several limitations. It relied on a survey that asked subjects about their dietary habits on average over a year coupled with objective laboratory results on a particular day. This might be expected to underestimate true correlations if the dietary effects are short term. Independent effects of vitamin C and E intake were not evaluated because of their high correlation. Importantly, it has been shown that those subjects using vitamin supplements are more likely to engage in healthy lifestyles, of which the factors are multivariate.55 Thus, it is possible that some factor(s) correlated with vitamin intake is actually influencing PON1 activity. The vitamin effects demonstrated in the present study are independent of any effect of current smoking and statin drug use, both of which influence PON1 activity. The vitamin effects on PON1 activity were also independent of dietary fat (calories) and alcohol (grams), neither of which predicted PON1 activities (results not shown). The present study suggests the need for clinical intervention tests evaluating causality and potential dosage effects, although the results of short-term and long-term supplementation may differ. Additionally, PON1 genotype distributions vary among ethnic groups,56 – 58 as may dietary effects on PON1 activity. Further studies of women are also warranted.

In summary, PON1 activity is predicted by antioxidant vitamin intake, smoking, and statin drug therapy, as well as by variation in the PON1 gene. We cannot rule out the possibility that the relationship between vitamin intake and PON1 activity is secondary to a correlated unmeasured healthy lifestyle, as discussed above. PON1 activity appears to be a better predictor of vascular disease than genotype for the PON1 coding region polymorphisms.5,10 For this reason, the noncoding genetic and environmental determinants of PON1 activity merit study as possible factors determining the role of PON1 in vascular disease. Furthermore, the role of PON1 and of dietary antioxidants in vascular disease may both be best understood if jointly considered.

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