Smoking Is Associated With Reduced Serum Paraoxonase Activity and Concentration in Patients With Coronary Artery Disease

Richard W. James, PhD; Ilia Leviev, PhD; Alberto Righetti, MD

Background—Paraoxonase is an HDL-associated enzyme that protects lipoproteins from oxidative modifications. Smoking is a major cardiovascular risk factor that promotes lipid peroxidation. Cigarette smoke has been shown in vitro to inhibit paraoxonase. The present study examined the hypothesis that smoking is associated with modulated serum activities and concentrations of paraoxonase.

Methods and Results—Coronary artery disease was assessed with the use of coronary arteriography in participants recruited from a hospital cardiology division. Medical and lifestyle data were obtained, and a fasting blood sample was provided. Three smoking categories were established (never, ex-smokers, and current smokers), and serum paraoxonase variables were compared among them. The activities and concentrations of paraoxonase were significantly lower in current than in never smokers. Ex-smokers had values comparable to those of never smokers. Ex-smokers who had recently stopped (<3 months) had activities and concentrations comparable to those of current smokers; values returned to the levels of never smokers within 2 years of cessation of smoking. Smoking status was an independent determinant of paraoxonase activity and concentration in multivariate analysis. Finally, lower paraoxonase was associated with more severe coronary disease and a reduced capacity to protect LDL from oxidation.

Conclusions—Smoking is independently associated with significant decreases in serum paraoxonase activities and concentrations, which normalize within a relatively short time of cessation. Lower serum paraoxonase is linked to more severe coronary artery disease and a lower antioxidant capacity. The data are consistent with the hypothesis that smoking modifies serum paraoxonase such that there is an increased risk of coronary artery disease due to a diminished capacity to protect lipoproteins from oxidative stress. (Circulation. 2000;101:2252-2257.)

Key Words: lipoproteins ■ smoking ■ coronary disease ■ antioxidants ■ risk factors

Paraoxonase (PON) is a serum enzyme that is entirely complexed to HDL.1 A recent hypothesis suggests an antioxidant role for PON in the protection of LDL from oxidative modifications.2 Substantial in vitro evidence supports such a function.3–4 This is complemented by studies of a recently developed PON knockout mouse model that demonstrated greater susceptibility of the lipoproteins to oxidation.5 The mice also developed more extensive atherosclerotic plaques, which is consistent with the hypothesis that oxidized lipoproteins play a major role in atherosclerosis.6 In humans, we identified PON as an independent, genetic risk factor for coronary artery disease (CAD).7,8 The observation has been confirmed in independent studies9–13 although not uniformly.14,15

Smoking is firmly established as 1 of the principal cardiovascular risk factors. Oxidative stress is considered to be the major, pathological mechanism associated with smoking, leading notably to lipid peroxidation.16–18 Several studies have demonstrated increased susceptibility of LDL to oxidation and higher levels of oxidized LDL in smokers.19–21 This would provide an important causal mechanism that links smoking with vascular disease given the numerous pathological effects of oxidized LDL.22,23

Smoking may enhance oxidative stress not only through the production of reactive oxygen radicals in smoke but also through weakening of the antioxidant defense mechanisms. In this context, a recent in vitro study showed that extracts of cigarette smoke inhibited the enzymatic activity of PON.24 Given its hypothesized, antioxidant role, this could also contribute to the increased oxidation of LDL in smokers. The present study examined the hypothesis that smoking is associated with lower serum PON activity and examined the possible consequences of lower PON levels with respect to the protection of LDL from oxidation and the severity of vascular disease.

Methods

Study Population

The present study was undertaken in cardiovascular patients and control subjects recruited from patients attending the Cardiology
Division of the University Hospital, Geneva. They gave written, informed consent to participate, and the study was performed according to the requirements of the ethical committee of the medical faculty. All participants underwent a biplane coronary arteriographic examination (standard Judkins technique), and the degree of stenosis was visually interpreted by a panel of experienced senior cardiologists. No other attempt was made to quantify the extent of the lesions. Arteriograms were considered positive for CAD (CAD+) if a stenosis estimated as >20% was detected in 1 major epicardial vessels. Patients were further classified as having 1-, 2-, or 3-vessel disease according to the presence of a stenosis of >70% in 1, 2, or 3 of the major epicardial vessels. Subjects with no detectable stenosis in any artery were considered free of disease (CAD−); patients with myocardial infarction were also excluded from the latter group. An analysis of the relationship of serum PON to disease severity was limited to control subjects with no detectable stenosis and patients with ≥1 lesion of >70%. The assessment of lesion severity and quantification of serum PON were completely independent; cardiologists were not aware of clinical data with respect to PON, whereas personnel who performed clinical analyses or interviewed patients were unaware of the arteriographic assessment.

Each participant completed questionnaires on lifestyle and personal and family medical history with a qualified interviewer. Patients were asked if they were current smokers or nonsmokers. Nonsmokers were classified as either never smokers or ex-smokers. Smoking Status

### Lipoproteins and Oxidation

For studies of lipoprotein oxidation, LDL (δ 1.019 to 1.063) and HDL (δ 1.063 to 1.21) were isolated with ultracentrifugation from fasting plasma and serum samples provided from a donor pool. Oxidation of LDL was performed as described previously with Cu²⁺ (5 μmol/L) as the oxidizing agent. Lipid hydroperoxides were quantified with the use of hydrogen peroxide to develop the calibration curve (intra-assay and interassay coefficients of variation were 1.0 and 2.2, respectively).

Protection assays were performed as described with the use of HDL that contained PON isolated by ultracentrifugation. Protection was determined in a comparison of lipid hydroperoxides formed in LDL+HDL coinulates (LDL and HDL mixed in a 1:2 protein ratio) with lipid hydroperoxides formed in LDL and HDL incubated individually. PON-free HDL was prepared with the passage of HDL through an affinity column that contained anti-PON antibodies. The non-bound fraction was collected and concentrated. It contained >95% of HDL protein and lipids; no PON enzyme activity could be detected in the fraction, and PON protein was absent on Western blotting. PON-free HDL was used to dilute PON-enriched HDL to lower the PON activity levels while maintaining lipid and total protein concentrations of HDL.

### Analyses

Fasting serum and plasma samples were obtained. Plasma was used to measure lipid, lipoprotein, and apolipoprotein levels as described previously. LDL cholesterol was calculated with the Friedewald formula. Serum samples were used to quantify PON enzyme activities and mass concentrations, as described previously. DNA was extracted from blood cells and genotyped for polymorphisms 191 and 54 of the PON1 gene.

### Statistical Analyses

Patients were categorized as never smokers, ex-smokers (stopped ≥1 week before recruitment), or current smokers. Comparisons between categories were performed with ANOVA. Variables that influence serum PON activities and concentrations were analyzed with forward stepwise regression analysis. Analyses were performed with the JMP statistical package (SAS Institute).

### Results

A total of 596 subjects were included in the study (Table 1). Mean lipid levels differed significantly among the 3 defined smoking categories with respect to HDL cholesterol, apolipoprotein A-I, and triglyceride levels. It should be noted that the percentage of women was significantly higher in the never smoked category, but when men alone were analyzed, similar results were obtained (results not shown). The frequency of CAD was significantly lower in the never smoked group but did not differ between ex-smoker and current smoker groups (Table 1). Regarding medication, 58% of the population took aspirin on a regular basis (a similar percentage of never smokers [49.6%] and current smokers [51.4%] but a higher percentage of ex-smokers [64.8%]; P<0.02). Lipid-lowering drugs were used by 33.7% of the population, with similar percentages in the 3 subgroups (never, ex, and current 30.1%, 37.4%, and 29.1%). HMG-CoA reductase inhibitors were predominantly used (81.3%), with a similar percentage of users in the 3 groups. Vitamin supplementation was infrequent (11.7%) and...
did not differ significantly among smoking categories (never, ex, and current 14.4%, 10.5%, and 12.0%).

Table 2 shows the serum activities and concentrations of PON as a function of smoking category. With both substrates, enzyme activities were lowest in the current smoker group. This was paralleled by significantly lower mass concentrations of PON in the smoker group (Table 2). Ex-smokers had values intermediate between those of never and current smokers. In a complementary evaluation, activities and concentrations of PON were analyzed as a function of the time since ex-smokers had stopped smoking. Ex-smokers (n = 75) who had stopped between 0.25 and 24 months before entry into the study showed a significant, positive, albeit weak, correlation between duration of nonsmoking and PON activity (phenylacetate, \( r = 0.25 \), \( \text{P} = 0.032 \)) and concentration (\( r = 0.25 \), \( \text{P} = 0.033 \)). Mean activities and concentrations were subsequently compared in ex-smokers who had stopped between 0.25 and 3 months (n = 40, mean 1.4 months) and those who had stopped between 3 and 24 months (n = 35, mean 12.4 months). In a comparison of current smokers (n = 142), ex-smokers (<3 months), ex-smokers (3 to 24 months), and never smokers (n = 139), there were significant differences in activity with phenylacetate (83.3 [1.7] versus 81.5 [2.6] versus 91.6 [4.7] versus 92.3 [2.7] U/mL; \( \text{P} = 0.012 \)) and for concentration (97.5 [1.6] versus 98.7 [3.2] versus 104.6 [3.1] versus 104.4 [2.0] \( \mu \text{g/mL} \); \( \text{P} = 0.028 \)). With paraoxon as substrate, the respective mean values were 248.4 (13.4) versus 314.1 (29.5) versus 352.3 (30.9) versus 291.5 (16.1) U/mL (\( \text{P} = 0.009 \)). Those who had stopped smoking for a longer period again showed higher activities. The higher activities with paraoxon in the ex-smoker groups compared with the never smokers reflects the higher percentage of BB homozygotes in the ex-smokers (15.0% versus 8.6%), with the B isoform showing increased activity with paraoxon. These results are consistent with smoking having a short-term rather than a long-term influence on serum PON.

Factors independently associated with variations in serum enzyme activities and concentrations of PON were analyzed with the use of stepwise regression analysis. These analyses also took into account polymorphisms 191 and 54 of the human PON gene, which have strong associations with serum PON activities and concentrations.\(^8,34\) Table 3 shows the best models that emerged from these analyses. Smoking status was significantly associated with variations in enzyme activity (phenylacetate) and concentration independent, notably, of sex, age, cardiovascular status (CAD+ or CAD−), diabetes, and sex.

**TABLE 2. Serum PON Activities and Concentrations as a Function of Smoking Status**

<table>
<thead>
<tr>
<th>Smoking Status</th>
<th>Never</th>
<th>Ex-Smoker</th>
<th>Current</th>
<th>( \text{P (3 Groups)} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylacetate, U/mL</td>
<td>92.3 (2.7) †</td>
<td>88.1 (1.4)*</td>
<td>83.3 (1.7)</td>
<td>0.014</td>
</tr>
<tr>
<td>Paraoxon, U/mL</td>
<td>291.5 (16.1)*</td>
<td>291.6 (10.9)*</td>
<td>248.4 (13.4)</td>
<td>0.05</td>
</tr>
<tr>
<td>Concentration, ( \mu \text{g/mL} )</td>
<td>104.4 (2.0) †</td>
<td>101.0 (1.1)</td>
<td>97.5 (1.6)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Mean (SEM) values are given. Paraoxon activity was measured under basal conditions. Comparisons were made with ANOVA.

\( ^* \text{P}<0.05 \) vs current smokers.

\( ^† \text{P}<0.01 \) vs current smokers.

**TABLE 3. Stepwise Regression Analysis of Factors Associated With Variations in Enzyme Activities and Serum Concentrations of PON**

<table>
<thead>
<tr>
<th>Independent Variable</th>
<th>Dependent Variable</th>
<th>( r^2 )</th>
<th>( \text{P} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arylesterase activity</td>
<td>apoA-I</td>
<td>11.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>3.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Genotype 54</td>
<td>3.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Genotype 191</td>
<td>1.84</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td></td>
<td>HDL cholesterol</td>
<td>0.67</td>
<td>0.026</td>
</tr>
<tr>
<td></td>
<td>Triglycerides</td>
<td>0.60</td>
<td>0.035</td>
</tr>
<tr>
<td></td>
<td>Smoking status</td>
<td>0.59</td>
<td>0.038</td>
</tr>
<tr>
<td>PON activity</td>
<td>Genotype 191</td>
<td>76.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>apoA-I</td>
<td>2.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>0.35</td>
<td>0.0019</td>
</tr>
<tr>
<td></td>
<td>HDL cholesterol</td>
<td>0.22</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>LDL cholesterol</td>
<td>0.15</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Triglycerides</td>
<td>0.15</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Smoking status</td>
<td>0.13</td>
<td>0.06</td>
</tr>
<tr>
<td>Concentration</td>
<td>Genotype 54</td>
<td>10.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Triglycerides</td>
<td>10.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>HDL cholesterol</td>
<td>7.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>LDL cholesterol</td>
<td>3.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>0.9</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>Smoking status</td>
<td>0.60</td>
<td>0.022</td>
</tr>
</tbody>
</table>

Other variables tested in all models were cardiovascular status (CAD+ or CAD−), diabetes, and sex.
an analysis of the physiological relevance of the reduction in serum PON with respect to its hypothesized role of protecting LDL from oxidation. The Figure, A, shows lipid hydroperoxides generated in LDL incubated with HDL containing increasing concentrations of PON, up to 20% of its mean normal concentration. There was a continual decrease in lipid hydroperoxides measured in LDL, showing that incremental changes in the PON content of HDL, of the order of differences in serum concentrations between never and current smokers, influence the ability of HDL to protect LDL from oxidation. The Figure, B, shows the protective capacity of HDL differing in PON concentrations by 10%. There were significantly higher levels of lipid hydroperoxides (P<0.02) in LDL incubated with the HDL containing lower concentrations of PON. Thus, control HDL (normal PON concentration) reduced LDL hydroperoxides by 50.0±3.5% after a 3-hour incubation, whereas HDL with lower PON reduced hydroperoxide levels by 35.6±3.6%. The difference was evident after longer incubation periods (Figure, B), reflecting previous studies that showed a more pronounced effect of PON as greater concentrations of lipid hydroperoxides are generated.30,35

### TABLE 4. Serum PON as a Function of Severity of CAD in Smokers and Ex-Smokers

<table>
<thead>
<tr>
<th>Disease Status</th>
<th>1 Vessel</th>
<th>2 Vessels</th>
<th>3 Vessels</th>
<th>P (3 Groups)</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>119</td>
<td>94</td>
<td>123</td>
<td></td>
</tr>
<tr>
<td>Activity phenylacetate, U/mL</td>
<td>90.7 (2.4)</td>
<td>86.9 (2.5)</td>
<td>82.0 (1.8)*</td>
<td>0.017</td>
</tr>
<tr>
<td>Activity paraoxon, U/mL</td>
<td>305.0 (18.1)</td>
<td>244.2 (16.7)*</td>
<td>261.2 (15.1)*</td>
<td>0.033</td>
</tr>
<tr>
<td>Concentration, μg/mL</td>
<td>104.0 (2.0)</td>
<td>98.7 (1.8)</td>
<td>97.7 (1.6)*</td>
<td>0.029</td>
</tr>
</tbody>
</table>

Mean (SEM) values are given. Patients with stenoses <70% or who had never smoked were excluded. Patients were categorized according to the presence of stenosis of >70% in 1, 2, or 3 major epicardial vessels. Comparable mean values (SEM) in CAD−subjects were phenylacetate 96.7 (4.4) U/mL, paraoxon 322.6 (29.8) U/mL, and concentration 105.2 (2.9) μg/mL.

Significance was tested with ANOVA: *P<0.05, †P<0.01 vs 1-vessel disease.

### Discussion

Cigarette smoking is firmly established as a primary risk factor for atherosclerotic, cardiovascular disease. Increased oxidative stress is 1 of the principal mechanisms by which it may exert its pathological influence.17 Available data furnish persuasive arguments for an antioxidant function of PON,2–4 which would represent an antiatherogenic potential of particular importance. In this context, the results presented here are highly consistent with the hypothesis that modifications of serum PON could be a mechanism by which smoking accelerates the atherogenic process. Serum PON activities assayed with 2 distinct substrates were significantly lower in current smokers compared with those in nonsmokers. Although there is debate concerning the extent to which activities measured with exogenous substrates (particularly paraoxon) reflect the activity of PON toward its physiological substrate,34–37 our demonstration that there also were significantly lower serum concentrations of PON in current smokers appears to eliminate any doubt arising from the use of possibly inappropriate substrates. Associations do not prove cause and effect, but several observations favor a direct relationship between smoking and serum PON. First, the associations were independent of other factors, notably diabetes, vascular disease, and sex. Diabetes may be associated with a lower activity of serum PON,38 whereas other studies39,40 (albeit not confirmed41) suggested a lower activity of serum PON in patients with myocardial infarction. PON is tightly linked to HDL and shows significant correlations with apoA-I concentrations.8,33 Diabetes and myocardial infarction are also associated with lower serum levels of HDL, whereas there is a well-established sex difference in HDL concentrations. Hence, variations in HDL associated with these factors could explain differences in PON activities. This does not appear to be the case, because smoking status was an independent predictor of PON activity variations in the presence of these factors and, more important, when HDL and apoA-I were included in the models. Vitamin supplementation, which could conceivably protect PON through augmentation of the overall antioxidant capacity, was low and similar among the 3 groups and thus would not appear to influence the results. There also was no significant difference between current and never smokers groups with respect to the use of aspirin and lipid-lowering medication. Second, smok-
ing status was associated with PON (arylesterase) activity and concentration independent of genetic polymorphisms 191 and 54. The association was of borderline significance for paraoxon, but the hugely predominant effect of the 191 polymorphism on this activity (76% of the variation) should be underscored. Third, in ex-smokers, activity and concentration values tended to revert to those of never smokers. Reduced activities and concentrations were present in ex-smokers who had recently stopped smoking (<3 months) but were recovered within 2 years. Finally, there is in vitro evidence of a direct inhibitory effect of cigarette smoke on PON enzyme activity,24 which is consistent with our observations.

The results of the present study further suggest that activity differences do not arise from the inhibition of enzyme activity alone. There was a decrease in PON protein; concentrations were significantly different in current smokers compared with never smokers, whereas ex-smokers had intermediate levels. In addition, we observed no significant differences in specific activities (enzyme activities per unit mass PON) among the 3 smoker categories, although values tended to be lower in current smokers (results not shown).

The evident question is whether the observations are of relevance to the occurrence of cardiovascular disease. Smoking has such a powerful impact on the risk of disease that it is difficult to dissociate a risk factor that may interact with it. As 1 approach, we analyzed PON in patients with differing severities of CAD, excluding never smokers. The rationale was that if serum PON were of relevance, lower levels could be observed in more severe cases of disease. The analysis demonstrated that enzyme activities and concentrations were significantly lower in patients with 3-vessel disease. Although this does not provide proof of a causal relationship, it is consistent with the accentuation of the risk of disease by a lower serum PON level. Of interest in this respect is a recent study that suggests that the link we previously demonstrated between the PON B allele and the risk of vascular disease7 may be exacerbated by smoking.13

More important, perhaps, we demonstrate that differences in PON concentrations, on the order of those observed in the present study, can influence the ability of HDL to protect LDL from oxidation. Thus, incremental increases in HDL PON are associated with incremental decreases in the level of LDL hydroperoxides generated under oxidation conditions. To complement this analysis, we also show that lowering the PON content of HDL by 10% was associated with a significantly higher concentration of LDL hydroperoxides. The studies were performed with PON-free HDL, which allowed us to maintain HDL protein and lipid concentrations while varying its PON content. We consider this important because several studies have shown that there are HDL-associated antioxidant activities that are independent of PON.42–45 The PON-enriched HDL subfraction removed through immunoaffinity chromatography contains only 2 other peptides (apoA-I and clusterin [apoJ],1 of which minor quantities were removed), and neither has been shown to have antioxidant enzymic activity.

In vitro studies are providing a wealth of data on the functions of PON, but observations that concern the clinical consequences of modifications to serum PON are less abun-

dant. A limited number of studies have reported lower PON activities in pathologies associated with a higher risk of vascular disease. PON has also been identified as a genetic risk factor for vascular disease. The present study extends these observations by showing (1) an association between smoking, a prooxidant phenomenon with a demonstrated inhibitory effect on PON, and serum PON activities and concentrations and (2) that recuperation of normal PON activities and concentrations coincides with the cessation of smoking. Our data also indicate that lower serum PON levels are associated with increased severity of CAD and reduced capacity to protect LDL from oxidation. They are consistent with the hypothesis that smoking modifies serum PON such that there is an increased risk of CAD, which may be due to a diminished capacity to protect lipoproteins from oxidative stress.

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References


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