Enhanced In Vivo Lipid Peroxidation at Elevated Plasma Total Homocysteine Levels


Abstract—An elevated plasma total homocysteine level (tHcy) is considered an independent risk factor for atherosclerosis. The mechanisms by which hyperhomocysteinemia induces atherosclerosis are only partially understood, but promotion of LDL oxidation and endothelial injury have been suggested. The purpose of this study was to test the hypothesis that a high plasma tHcy is associated in men with increased in vivo lipid peroxidation, as measured by plasma F2-isoprostane concentrations. We investigated this association in a subset of the participants in the Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) study. Of 256 male participants, a subsample of 100 consecutive men was selected for F2-isoprostane assays. The mean tHcy was 11.0 μmol/L, and the mean F2-isoprostanes was 29.6 ng/L. The simple correlation coefficient for association between tHcy and F2-isoprostane was 0.40 (P < 0.001). In a linear regression model, the variables with the strongest associations with F2-isoprostane were tHcy (standardized coefficient 0.33, P < 0.001), serum triglycerides (0.21, P = 0.042), carbohydrate-deficient transferrin (0.15, P = 0.132), and plasma lipid-standardized α-tocopherol (−0.11, P = 0.252) (R² = 0.24, P < 0.001 for model). Plasma F2-isoprostane levels increased linearly across quintiles of tHcy (P < 0.001). The unadjusted mean (95% confidence interval) F2-isoprostanes was 47.5% greater in the highest tHcy quintile (37.4, 31.1 to 43.6 ng/L) than in the lowest quintile (25.3, 21.3 to 29.3 ng/L). Adjustment for the strongest other determinants of F2-isoprostane reduced this difference to 28.2% (P = 0.010). Our present data suggest that elevated fasting plasma tHcy is associated with enhanced in vivo lipid peroxidation in men. (Arterioscler Thromb Vasc Biol. 1999;19:1263-1266.)

Key Words: homocysteine ■ F2-isoprostanes ■ lipid peroxidation ■ cardiovascular diseases

An elevated plasma total homocysteine level (referred to as tHcy), the sum of all homocysteine species in plasma, including free and protein-bound forms, has been suggested as an independent risk factor for atherosclerosis.1 Several studies have demonstrated elevated plasma tHcy levels in patients with coronary, cerebrovascular, or peripheral vascular diseases, compared with controls. This association is strong and independent of other risk factors for atherosclerosis, and it is not only limited to severe hyperhomocysteinemia but observed also when tHcy is only moderately elevated.2 The mechanisms by which hyperhomocysteinemia may induce atherogenesis are only partially understood,2 but promotion of LDL oxidation and endothelial injury have been suggested.

There is evidence from epidemiologic studies that implicates a role for lipid peroxidation in atherogenesis.3,4 Standardized methods for the assessment of lipid peroxidation in vivo have been lacking. F2-isoprostanes are a recently described class of prostaglandin-like compounds that are produced by free radical–mediated lipid peroxidation of arachidonic acid independent of cyclooxygenase.5 The quantification of F2-isoprostanes in plasma has proven to be a valuable method in assessing lipid peroxidation in vivo.6,7 Although epidemiological evidence about the association between plasma F2-isoprostanes and cardiovascular diseases is limited, increased amounts of F2-isoprostanes have been found in human atherosclerotic lesions when compared with nonatherosclerotic vessel walls.8,9 Alfthan et al10 reported an association between mean plasma tHcy concentration and mortality from cardiovascular disease (CVD) in WHO Monitoring of Trends and Determinants of Cardiovascular Disease (MONICA) data. Their data show that cross-country differences in plasma tHcy concentrations are real and not caused by noncomparable sampling and assay procedures. The correlation coefficient for plasma tHcy and CVD mortality was 0.71. The highest CVD mortality and mean plasma tHcy concentration both were observed in Finland.

The purpose of this study was to test the hypothesis that plasma tHcy concentration is associated in men with enhanced lipid peroxidation in vivo, as measured by plasma F2-isoprostane concentrations.

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Methods

We studied this association in a subset of the participants in the Antioxidant Supplementation in Atherosclerosis Prevention (ASAP) study.\textsuperscript{11} The ASAP study is a balanced 2×2 factorial double-masked placebo-controlled randomized clinical trial to study the effects of vitamin C and E supplementation on oxidative stress, lipid peroxidation, atherosclerotic progression, blood pressure, and cataract formation in humans. The study protocol was approved by the Research Ethics Committee of the University of Kuopio. From 256 male participants, a subsample of 100 consecutive men were selected for F\textsubscript{2}-isoprostane assays.

For the present study, all measurements were done at the baseline visit between April and October 1995. The age of these men was 58.6±6.5 years (mean±SD), and their serum total cholesterol was 6.14±0.93 mmol/L.

Subjects were instructed to abstain from eating for 12 hours and from ingesting alcohol for a week. After a subject had rested in sitting position for 5 minutes, blood was drawn into Venoject vacuum tubes (Terumo Corp). On the day before blood drawing, a 24-hour urine was collected.

Plasma F\textsubscript{2}-isoprostane concentrations were determined at Vanderbilt University Medical Center, Nashville, Tenn, in 1997. A deuterated prostaglandin F\textsubscript{2}\textalpha\textsubscript{a} internal standard was added to plasma, and F\textsubscript{2}-isoprostanes were extracted with C\textsubscript{18} and silica minicolumns. Compounds were converted to pentafluorobenzyl ester trimethylsilyl ether derivatives and analyzed by gas chromatographic–mass spectrometric assay.\textsuperscript{12} The coefficient of variation for both intraday and interday variation was 9%.

Plasma tHcy concentrations were measured at the National Public Health Institute, Helsinki, Finland, essentially as described by Araki and Sako.\textsuperscript{11} Homocysteine, other mixed disulfides, and the protein-bound homocysteine were first reduced to free homocysteine by tributylphosphine, and tHcy was measured using an isocratic reversed-phase HPLC method with fluorescence detection as described earlier in detail.\textsuperscript{11} The coefficient of variation between series was 7%.

Plasma a-tocopherol and \beta-carotene were determined by HPLC, and serum lipids were quantified photometrically at the Research Institute of Public Health, Kuopio, Finland, as described earlier.\textsuperscript{11} Urinary nicotine metabolite 24-hour excretion was measured by a colorimetric method (Enilab). To separate the effect of tHcy from that of serum lipids, values of lipid-standardized \alpha-tocopherol and \beta-carotene were first reduced to free homocysteine by tributylphosphine, and tHcy was measured using an isocratic reversed-phase HPLC method with fluorescence detection as described earlier in detail.\textsuperscript{11} The coefficient of variation between series was 7%.

The measurement of serum carbohydrate-deficient transferrin (CDT) is based on alcohol-altered glycosylation process of transferrin. Resulting transferrin is missing carbohydrate–mass spectrometric assay.\textsuperscript{12} The measurement of carbohydrate–mass spectrometric assay.\textsuperscript{12} The measurement of carbohydrate–mass spectrometric assay.\textsuperscript{12} The statistical significance of a linear trend in plasma F\textsubscript{2}-isoprostane concentration over quintiles of tHcy was tested using SPSS 1-way ANOVA, with 95% confidence intervals (CI) estimated taking into account the number of tHcy categories. A stepwise linear multivariate regression analysis was used to find the strongest other determinants of F\textsubscript{2}-isoprostane concentration in quintiles of tHcy categories. A stepwise linear multivariate regression analysis was used to find the strongest other determinants of F\textsubscript{2}-isoprostane concentration in quintiles of tHcy categories. A stepwise linear multivariate regression analysis was used to find the strongest other determinants of F\textsubscript{2}-isoprostane concentration in quintiles of tHcy categories. A stepwise linear multivariate regression analysis was used to find the strongest other determinants of F\textsubscript{2}-isoprostane concentration in quintiles of tHcy categories. A stepwise linear multivariate regression analysis was used to find the strongest other determinants of F\textsubscript{2}-isoprostane concentration in quintiles of tHcy categories. A stepwise linear multivariate regression analysis was used to find the strongest other determinants of F\textsubscript{2}-isoprostane concentration in quintiles of tHcy categories. A stepwise linear multivariate regression analysis was used to find the strongest other determinants of F\textsubscript{2}-isoprostane concentration in quintiles of tHcy categories.

Results

The mean plasma tHcy was 11.0 μmol/L, and the mean of F\textsubscript{2}-isoprostanes 29.6 ng/L. The simple correlation coefficient for association between plasma tHcy and F\textsubscript{2}-isoprostane was 0.40 (\textit{P}<0.001). This correlation was 0.40 (\textit{P}=0.003) in 50 men whose serum total cholesterol was below the median (6.07 mmol/L) and 0.42 (\textit{P}=0.003) in 50 men with a higher serum cholesterol. In a linear regression model, the variables with the strongest associations with F\textsubscript{2}-isoprostanes were tHcy (standardized coefficient 0.33, \textit{P}<0.001), serum triglycerides (0.21, \textit{P}=0.042), CDT (0.15, \textit{P}=0.132), and plasma lipid-standardized \alpha-tocopherol (−0.11, \textit{P}=0.252) (\textit{R}²=0.24, \textit{P}<0.001 for model) (Table).

Plasma F\textsubscript{2}-isoprostane levels increased linearly across the quintiles of plasma tHcy (\textit{P}<0.001 for linear trend, Figure). The unadjusted mean (95% CI) of F\textsubscript{2}-isoprostanes was 47.5% greater in the highest tHcy quintile (37.4, 31.1 to 43.6 ng/L) than in the lowest quintile (25.3, 21.3 to 29.3 ng/L). Adjustment for the strongest other determinants of F\textsubscript{2}-isoprostane reduced this difference to 28.2% (\textit{P}=0.010). In the multiple range test, the F\textsubscript{2}-isoprostane mean was significantly (\textit{P}<0.05) higher in the 2 highest tHcy quintiles compared with the lowest quintile. History of smoking or the proportion of men with diabetes mellitus did not differ significantly between plasma tHcy quintiles.

We also compared the group with an elevated tHcy (>12 μmol/L) with the low tHcy group (≤12 μmol/L) with and without adjustment for the strongest other determinants of F\textsubscript{2}-isoprostane. The unadjusted mean (95% CI) F\textsubscript{2}-isoprostane concentration in quintiles of plasma tHcy concentration in 100 men from eastern Finland.

The white bars denote unadjusted means and the gray bars, means that are adjusted for serum triglycerides, plasma \beta-carotene, serum CDT, and plasma lipid-standardized \alpha-tocopherol levels and urinary nicotine metabolite excretion.

<table>
<thead>
<tr>
<th></th>
<th>Unadjusted Regression Coefficient</th>
<th>Adjusted Regression Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma homocysteine, μmol/L</td>
<td>0.379*</td>
<td>0.334*</td>
</tr>
<tr>
<td>Serum triglyceride, mmol/L</td>
<td>0.174</td>
<td>0.214†</td>
</tr>
<tr>
<td>Serum CDT, U/L</td>
<td>0.187</td>
<td>0.149</td>
</tr>
<tr>
<td>Plasma lipid-standardized \alpha-tocopherol</td>
<td>−0.085</td>
<td>−0.109</td>
</tr>
<tr>
<td>Plasma \beta-carotene, μmol/L</td>
<td>−0.222†</td>
<td>−0.105</td>
</tr>
<tr>
<td>Urinary nicotine metabolite excretion, μmol/d</td>
<td>0.166</td>
<td>0.086</td>
</tr>
</tbody>
</table>

\textit{R}² for the model (including also time of drawing the blood sample), 0.245; \textit{P}=0.0002. Coefficients are standardized to SD. *\textit{P}<0.001. †\textit{P}<0.05.
isoprostanes were 34% greater in the high tHcy group (36.0, 31.0 to 41.0 ng/L) compared with the low tHcy group (26.8, 24.3 to 29.2 ng/L; P<0.001). Adjustment for other strongest determinants shown in the Table reduced this difference to 22.1% (P=0.018).

**Discussion**

To our knowledge, this is the first study to show that a high fasting plasma tHcy is associated with enhanced lipid peroxidation in vivo in men. Lipid peroxidation was assessed in this study by quantifying plasma F₂-isoprostanes. These are prostaglandin-like products of nonenzymatic lipid peroxidation. Measurement of endogenous unmetabolized F₂-isoprostanes has proven to be a valuable approach to assess lipid peroxidation in vivo. The method we used to quantify F₂-isoprostanes measures the peak that coelutes with 8-iso-prostaglandin F₂α, but may contain other isoprostanes as it is impossible to cleanly separate various isoprostane compounds using this assay. Morrow and Roberts have shown that quantification of this peak correlates with total isoprostane production in all situations they have examined and is thus an accurate index of lipid peroxidation.

Although both retrospective and prospective epidemiological studies have shown that even moderate hyperhomocysteinemia is associated with an increased risk for premature vascular disease in the coronary, cerebral, and peripheral arteries, the risk-increasing mechanisms of the effect of plasma tHcy are still poorly understood. Homocysteine is readily oxidized when added to plasma, principally as a consequence of auto-oxidation leading to the formation of homocysteine, homocysteine-mixed disulfides, and homocysteine thiolactone. It has been proposed that during the transition metal ion-catalyzed oxidation of the sulphydryl group of homocysteine, hydrogen peroxide is formed, which promotes oxidative stress and lipid peroxidation through Fenton-type reactions. Formed hydrogen peroxide may also have direct harmful effects on the vascular endothelium. Homocysteine has also been shown to oxidize LDLs by reactions requiring redox-active transition metal ions. There is some evidence from animal models that supports the role of tHcy in lipid peroxidation. Young et al. induced hyperhomocysteinemia in 8 pigs by intermittent exposure to nitrous oxide for 4 weeks and compared these with air-breathing control animals. At necropsy they measured cardiac tissue maltoldehyde and unsaturated fatty acid concentrations and concluded that hyperhomocysteinemia was associated with increased in vivo lipid peroxidation. In these hyperhomocysteinemic animals, plasma tHcy levels were much higher than in moderately hyperhomocysteinemic men. Durand et al. studied acute methionine load-induced hyperhomocysteinemia and lipid peroxidation using a rat model. They found that a moderate hyperhomocysteinemia plays a role in the development of a thrombogenic state that might be mediated by the occurrence of lipid peroxidation, measured by an elevation of plasma conjugated dienes, lipid hydroperoxides, and thiobarbituric acid-reactive substances (TBARS).

Recently Domagala et al. published a study about hyperhomocysteinemia in which lipid peroxidation was measured using the oral methionine-loading test. Study subjects were 46 men and women with normal or pathological methionine-loading test result. They reported a significant relationship between plasma homocysteine levels and lipid peroxidation, expressed as an increase in TBARS. Our results at fasting state support these findings. Because some TBARS may be formed during the assay itself, measurement of endogenous unmetabolized F₂-isoprostanes is probably a more reliable measurement of in vivo lipid peroxidation.

In conclusion, the present study provides the first evidence of a role for elevated fasting plasma tHcy in lipid peroxidation in vivo in men and suggests a plausible mechanism through which an elevated plasma tHcy could increase the risk of atherothrombotic CVD.

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**References**