Oxidative Stress and Platelet Activation in Homozygous Homocystinuria

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Background—Severe hyperhomocysteinemia due to cystathionine β-synthase deficiency (CβSD) is associated with early atherothrombotic vascular disease. Homocysteine may exert its effects by promoting oxidative damage. In the present study, we investigated whether in vivo formation of 8-iso-prostaglandin (PG) F2α, a platelet-active product of arachidonic acid peroxidation, is enhanced in CβSD and whether it correlates with in vivo platelet activation, as reflected by thromboxane (TX) metabolite excretion.

Methods and Results—Urine and blood samples were obtained from patients with homozygous CβSD (n=13) and age-matched healthy subjects. Urinary 8-iso-PGF2α excretion was significantly higher in CβSD patients than in control subjects (640±384 versus 213±43 pg/mg creatinine; P=0.0015) and correlated with plasma homocysteine (ρ=0.398, P=0.0076). Similarly, urinary 11-dehydro-TXB2 excretion was enhanced in CβSD (1166±415 versus 324±72 pg/mg creatinine; P=0.0015) and correlated with urinary 8-iso-PGF2α (ρ=0.362, P=0.0153). Vitamin E supplementation (600 mg/d for 2 weeks) was associated with a statistically significant increase in its plasma levels (from 16.6±4.6 to 40.4±8.7 μmol/L, P=0.0002) and with reductions in 8-iso-PGF2α (from 790±159 to 559±111 pg/mg creatinine, P=0.018) and 11-dehydro-TXB2 (from 1273±383 to 913±336 pg/mg creatinine, P=0.028). A statistically significant inverse correlation was found between urinary 8-iso-PGF2α and plasma vitamin E levels (ρ=−0.745, P=0.0135).

Conclusions—The results of the present study suggest that enhanced peroxidation of arachidonic acid to form bioactive F2-isoprostanes may represent an important mechanism linking hyperhomocysteinemia and platelet activation in CβSD patients. Moreover, they provide a rationale for dose-finding studies of vitamin E supplementation in this setting. (Circulation. 2001;104:1124-1128.)

Key Words: homocystinuria ▪ platelets ▪ thromboxane ▪ isoprostanes ▪ lipids

Although the molecular mechanism(s) by which elevated levels of homocysteine (Hcy) favor atherothrombosis are at present poorly understood, the epidemiological evidence of an association between hyperhomocysteinemia and atherothrombotic vascular disease is quite consistent.1,2 Patients with severe hyperhomocysteinemia and homocystinuria develop occlusive vascular disease in early adulthood or even in childhood, whereas patients with mild hyperhomocysteinemia develop premature coronary artery disease, as well as recurrent arterial and venous thrombosis.1,2

In homocystinuric patients, homozygotes for mutations of the gene coding for cystathionine β-synthase (CBS), abnormalities of coagulation markers, and in vivo platelet activation have been reported.3,4 Enhanced TX biosynthesis of patients with CBS deficiency (CBSβSD) was not affected by a bolus infusion of hirudin that determined a long-lasting impairment of the conversion of fibrinogen to fibrin by thrombin.4 In contrast, in vivo platelet activation was reduced, at least in part, by the administration of the antioxidant drug probucol in this setting.3,4

Hyperhomocysteinemia is thought to exert its effects through a mechanism involving oxidative damage.5 During the auto-oxidation of Hcy in plasma, leading to the formation of homocystine, reactive oxygen species (superoxide anion radical and hydrogen peroxide) are generated. These oxygen-derived molecules are potentially responsible for endothelial dysfunction associated with hyperhomocysteinemia.5,6 Reactive oxygen species can initiate lipid peroxidation on the endothelial cell surface as well as within lipoprotein particles in plasma.5 In turn, oxidized LDL may trigger platelet activation as well as some of the hemostatic abnormalities reported in association with hyperhomocysteinemia. Thus, the oxidative modifications induced by Hcy may contribute to the pathogenesis of atherothrombosis in clinical syndromes of hyperhomocysteinemia.

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**TABLE 1. Clinical, Genetic, and Metabolic Features of the Homozygous CβSD Patients**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age at Diagnosis, y</th>
<th>Duration of Treatment, y</th>
<th>B6 Responsive</th>
<th>thcy, μmol/L</th>
<th>Vascular Disease</th>
<th>Plasma Vitamin E, μmol/L</th>
<th>Gene Mutations</th>
</tr>
</thead>
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<tr>
<td>S.R.</td>
<td>F</td>
<td>6</td>
<td>24</td>
<td>Yes</td>
<td>47.3</td>
<td>-</td>
<td>NA</td>
<td>C262T</td>
</tr>
<tr>
<td>S.S.</td>
<td>F</td>
<td>3</td>
<td>23</td>
<td>Yes</td>
<td>77.6</td>
<td>+</td>
<td>12.7</td>
<td>C262T</td>
</tr>
<tr>
<td>D.A.M.</td>
<td>F</td>
<td>10</td>
<td>19</td>
<td>Yes</td>
<td>17.1</td>
<td>+</td>
<td>16.4</td>
<td>C341T</td>
</tr>
<tr>
<td>D.A.</td>
<td>M</td>
<td>12</td>
<td>19</td>
<td>Yes</td>
<td>20.7</td>
<td>-</td>
<td>13.7</td>
<td>C341T</td>
</tr>
<tr>
<td>S.A.</td>
<td>M</td>
<td>8</td>
<td>6</td>
<td>Yes</td>
<td>123</td>
<td>-</td>
<td>NA</td>
<td>T833C</td>
</tr>
<tr>
<td>O.E.</td>
<td>M</td>
<td>25</td>
<td>26</td>
<td>Yes</td>
<td>39.6</td>
<td>-</td>
<td>21.9</td>
<td>C341T</td>
</tr>
<tr>
<td>S.G.</td>
<td>F</td>
<td>2</td>
<td>16</td>
<td>Yes</td>
<td>79.8</td>
<td>-</td>
<td>10.8</td>
<td>C262T</td>
</tr>
<tr>
<td>M.L.</td>
<td>M</td>
<td>3</td>
<td>25</td>
<td>Yes</td>
<td>238</td>
<td>-</td>
<td>19.6</td>
<td>G346A</td>
</tr>
<tr>
<td>M.A.</td>
<td>F</td>
<td>6</td>
<td>28</td>
<td>Yes</td>
<td>103.5</td>
<td>-</td>
<td>15.9</td>
<td>G346A</td>
</tr>
<tr>
<td>T.G.</td>
<td>M</td>
<td>4</td>
<td>22</td>
<td>No</td>
<td>143</td>
<td>+</td>
<td>12.8</td>
<td>C172T + C341T</td>
</tr>
<tr>
<td>P.V.</td>
<td>M</td>
<td>28</td>
<td>10</td>
<td>Yes</td>
<td>21.2</td>
<td>+</td>
<td>13.5</td>
<td>C146T + 1224-89del49†</td>
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<tr>
<td>D.M.G.</td>
<td>M</td>
<td>49</td>
<td>1</td>
<td>Yes</td>
<td>265</td>
<td>+</td>
<td>17.1</td>
<td>NA</td>
</tr>
<tr>
<td>D.M.F.</td>
<td>F</td>
<td>43</td>
<td>1</td>
<td>Yes</td>
<td>287</td>
<td>-</td>
<td>11.6</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA indicates not available.

*The allele was negative for 4 frequent (C341T; T833C; G904A; A1224-2C) and for 2 private (G393C and C434T) mutations.

†Htrton 11.

F₂-isoprostanes represent a family of bioactive prostaglandin (PG) F₂-like compounds that are produced from arachidonic acid through a nonenzymatic process of lipid peroxidation, catalyzed by oxygen free radicals on cell membranes and LDL particles (reviewed by Patrono and FitzGerald). Although cyclooxygenase-dependent mechanisms of isoprostane formation have been described, the relative contribution of these enzymatic pathways appears to be negligible in humans. Among F₂-isoprostanes, of particular interest is 8-iso-PGF₂α, which induces vasoconstriction and modulates the function of human platelets. F₂-isoprostanes can be reliably measured in both plasma and urine and have been reported to be increased in association with several cardiovascular risk factors, including advanced age, hypercholesterolemia, diabetes mellitus, and cigarette smoking.

We have previously reported biochemical evidence of persistent platelet activation, as reflected by enhanced generation of 8-iso-PGF₂α and 11-dehydro-TXB₂ excretion in CβSD patients. We speculated that increased oxidant stress in homozygous homocystinuria would induce enhanced generation of 8-iso-PGF₂α and other biologically active isoeicosanoids and that these compounds would in turn contribute to platelet activation in this setting. Therefore, in the present study, we investigated whether 8-iso-PGF₂α formation is altered in CβSD patients compared with age-matched healthy subjects and whether it correlates with plasma Hcy levels and with the rate of TXA₂ biosynthesis. Moreover, we examined the effects of short-term vitamin E supplementation by assessing time-related changes in urinary 8-iso-PGF₂α and 11-dehydro-TXB₂ excretion in this setting.

The results of the present study suggest that enhanced peroxidation of arachidonic acid to form biologically active isoprostanes may represent an important biochemical link between hyperhomocysteinemia and persistent platelet activation.

**Methods**

**Subjects**

Thirteen patients with homozygous CβSD (7 male, 6 female, age 14 to 51 years, Table 1), belonging to 8 unrelated families, were studied on several occasions. Homozygous CβSD patients exhibited abnormally high levels of plasma methionine and total Hcy (thcy) (with extremely low levels of cysteine), homocystinuria, ectopia lentis, osteoporosis, and different degrees of mental retardation. All but 1 were responsive to pyridoxine and had been on pyridoxine treatment (600 to 900 mg/d) from the time of diagnosis. The pyridoxine-unresponsive subject was on 8 g/d betaine. Leukocyte DNA analysis showed that 13 individuals (S.R., S.S., and S.G.) were compound heterozygotes for the mutations C262T and T833C. Genetic data concerning these individuals have been reported in detail elsewhere. For all the data reported in the present study, no differences were found between the 3 subjects with the C262T and T833C mutations and the other 10 patients. Thirteen apparently healthy individuals, age-matched with the homocystinuric patients, were also studied (Table 2). None of them had a history of diseases known to alter platelet function. No current use of drugs affecting platelet function was reported by any of the control subjects. CβSD patients were comparable to control subjects with respect to renal function, cigarette smoking, systolic and diastolic blood pressure, plasma glucose, and cholesterol and triglyceride levels. None of the

**TABLE 2. Characteristics of Patients With Homozygous CβSD and Control Subjects**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients (n=13)</th>
<th>Control Subjects (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex, M/F</td>
<td>7/6</td>
<td>7/6</td>
</tr>
<tr>
<td>Age, y (mean±SD)</td>
<td>28±14</td>
<td>29±12</td>
</tr>
<tr>
<td>BMI, kg/m² (mean±SD)</td>
<td>23±2.8</td>
<td>23±2.8</td>
</tr>
<tr>
<td>Smokers, n</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Cholesterol &gt;240 mg/dL, n</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Diabetes, n</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hypertension, n</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
CJBSD patients showed ST depression or Q waves on the ECG, and their clinical records were negative for angina pectoris, myocardial infarction, and venous thromboembolism. All had normal peripheral pulses and were negative for bruits over the carotid vessels. Duplex scanner analysis confirmed the negativity with respect to carotid arteries, whereas it revealed early signs of iliac stenosis (patient P.V.) or ankle/arm ratio <0.85 in 3 patients (S.S., D.A.M., and T.G.). Patient S.S. also exhibited a chronic vein insufficiency. Informed consent was obtained from all patients and volunteers after approval of the protocols by the local institutional review board. The procedures followed were in accordance with institutional guidelines.

**Design of the Studies**

In the first study, a cross-sectional comparison of urinary 8-iso-PGF$_{2\alpha}$ and 11-dehydro-TXB$_2$, a major enzymatic metabolite of TXA$_2$, was performed between patients and control subjects. All the subjects were studied as outpatients after a 12-hour fast. Blood samples were obtained in the morning. Each subject performed an overnight urine collection before blood sampling. Urine samples were added with the antioxidant 4-hydroxy-Tempo (1 mmol/L) (Sigma Chemical Co) and stored at −20°C until extraction.

To investigate the short-term effects of antioxidant intervention on urinary 8-iso-PGF$_{2\alpha}$ and 11-dehydro-TXB$_2$ excretion, vitamin E supplementation was performed in 7 (3 women and 4 men, age 18 to 51 years) of the same CJBSD patients. They were given L-α-tocopherol acetate 600 mg/d (Evanon) for 2 weeks after a baseline evaluation. Before and after vitamin E supplementation, they were instructed to perform an overnight urine collection, and a fasting sample of peripheral venous blood was drawn the following morning.

**Hcy and Vitamin E Determinations**

Fasting plasma tHcy (ie, the sum of free and protein-bound forms plus cysteine-homocysteine mixed disulfide) was obtained after cleavage and reduction reactions with sodium borohydride followed by iodoacetic acid treatment. High-performance liquid chromatography isolation of Hcy was carried out by the O-phthalaldehyde derivatization method and appropriate solvent systems (BDH). According to previously validated procedures, a fluorescence system was used to determine tHcy in the system. 6

**Vitamin E plasma levels were measured by high-performance liquid chromatography. 14**

**Urinary Eicosanoid Assays**

Urinary 8-iso-PGF$_{2\alpha}$ and 11-dehydro-TXB$_2$ were measured by previously described radioimmunoassay methods. 13,19 Measurements of urinary 8-iso-PGF$_{2\alpha}$ and 11-dehydro-TXB$_2$ by these methods have been validated with different antisera and by comparison with gas chromatography/mass spectrometry, as detailed elsewhere. 13,19

**Statistical Analysis**

The data were analyzed by nonparametric methods to avoid assumptions about the distribution of the measured variables. An ANOVA was performed with the Kruskal-Wallis method. Subsequent pairwise comparisons were made with the Mann-Whitney U test. The differences between baseline and posttreatment values were analyzed with the Wilcoxon signed-rank test. Moreover, the association of eicosanoid measurements with other biochemical parameters was assessed by the Spearman rank correlation test. All values are reported as mean±SD.

**Results**

As shown in Table 1, Hcy plasma levels showed marked interindividual variability in the 13 CJBSD patients. These levels are the mean of 2 to 5 repeated measurements. Over a 24-month period, ≥3 repeated urine samples obtained from 8 CJBSD patients yielded an intrasubject coefficient of variation of 47±19%. Moreover, consistent with the concept that such variations are primarily related to genetic factors affecting sensitivity to treatment as well as to differences in compliance with treatment of individual patients and/or their families, 3,18 similarities in the plasma Hcy levels of different members of the same family were observed (eg, patients S.S., S.G., D.A.M., and D.A. in Table 1).

**Urinary 8-iso-PGF$_{2\alpha}$ excretion was significantly (P=0.0015) higher in CJBSD patients (640±384 pg/mg creatinine, mean±SD, n=13) than in age-matched healthy subjects (213±43 pg/mg creatinine). All but 3 patients had excretion rates >2 SD above the control mean. Over a 24-month period, ≥3 repeated urine samples obtained from 8 CJBSD patients yielded an intrasubject coefficient of variation of 50±20%. A statistically significant correlation was found between plasma Hcy and urinary 8-iso-PGF$_{2\alpha}$ in CJBSD patients (ρ=0.398; n=46, P=0.0076) (Figure 1). Consistent with previous findings, 3 CJBSD patients had significantly enhanced excretion of 11-dehydro-TXB$_2$ versus healthy subjects (1166±415 versus 324±72 pg/mg creatinine; P=0.0015). Over a 24-month period, ≥3 repeated urine samples obtained from 8 CJBSD patients yielded an intrasubject coefficient of variation of 45±14%. A statistically significant correlation was found between urinary excretion of 8-iso-PGF$_{2\alpha}$ and 11-dehydro-TXB$_2$ in CJBSD patients (ρ=0.362, P=0.0153) (Figure 2).

**Effects of Vitamin E Supplementation**

We also examined the effects of vitamin E supplementation (600 mg/d for 2 weeks) on the urinary excretion of 8-iso-PGF$_{2\alpha}$ and 11-dehydro-TXB$_2$ in 7 CJBSD patients to test the hypothesis of a cause-and-effect relationship between enhanced lipid peroxidation and platelet activation in this
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found in our CβSD patients. Metabolite excretion was measured at baseline, at end of 2-week dosing with vitamin E 600 mg/d, and 1 month after vitamin E withdrawal. Dots and lines connecting dots represent measurements performed in individual patients.

Discussion

This is the first study to establish a biochemical link between enhanced lipid peroxidation and platelet activation in vivo in the setting of markedly elevated plasma levels of tHcy due to CβSD. Patients with this rare, recessively inherited disorder of methionine metabolism develop atherothrombotic vascular disease in childhood and adolescence.4 Previous studies have described enhanced in vivo lipid peroxidation in association with elevated plasma tHcy levels even within the normal range for Hcy.21 Moreover, heterozygous CβSD mice with mild hyperhomocysteinemia manifest evidence for enhanced lipid peroxidation.22 Our study extends this observation to a much wider range of plasma tHcy levels. Although the simple correlation coefficient for the association between plasma tHcy and F2-isoprostane production found in our CβSD patients is virtually identical to that found in men participating in the ASAP study,21 the slope of the correlation in the present study is rather shallow. This may reflect the increased circulating levels of extracellular superoxide dismutase, an important antioxidant in vascular tissue, recently described in association with high tHcy in CβSD patients.23 The latter could represent a protective antioxidant response to Hcy-induced oxidative damage in the vasculature.23

It should be noted that the 2 highest values of urinary 8-iso-PGF2α excretion measured in association with plasma tHcy >200 μmol/L are comparable to the highest values of this F2-isoprostane previously described in association with hypercholesterolemia14 and cystic fibrosis.24 Measurement of unmetabolized F2-isoprostanes in plasma and urine has proved to be a valuable approach to assess the actual rate of lipid peroxidation in vivo (reviewed in Reference 8).

The present study, we have characterized a novel mechanism whereby hyperhomocysteinemia may cause persistent platelet activation, ie, through free radical–catalyzed peroxidation of arachidonic acid to form the platelet-active F2-isoprostane 8-iso-PGF2α and possibly other isoeicosanoids. The results of a preliminary short-term intervention with vitamin E supplementation are consistent with a causal relationship between enhanced formation of 8-iso-PGF2α and in vivo TXA2 biosynthesis, a noninvasive biochemical index of platelet activation.19 In fact, the amount of 11-dehydro-TXB2 inhibition associated with the reduction of 8-iso-PGF2α formation after vitamin E supplementation is consistent with the theoretical change predicted by the linear correlation between the two, as depicted in Figure 2.

Although the results of the vitamin E supplementation study are mechanistically informative on the origin and biological activity of 8-iso-PGF2α in the setting of CβSD, they do not address the question of optimal dosing regimen and duration of such intervention. They provide useful information, however, for a formal sample-size calculation for a dose-finding study of vitamin E supplementation in this setting. Moreover, these preliminary observations suggest a potential role for antioxidant therapy in attenuating Hcy-dependent oxidative changes that may promote atherothrombosis in CβSD.

The findings in CβSD patients confirm and extend similar observations made in other clinical settings, such as hypercholesterolemia,14 diabetes mellitus,15 and cigarette smoking.16,17 Thus, regardless of the mechanism(s) responsible for enhanced lipid peroxidation, there is quite convincing evidence from studies in such diverse conditions that enhanced generation of bioactive isoeicosanoids may transduce the oxidant signal associated with a variety of cardiovascular risk factors into a functional platelet response that may contribute to enhanced thrombotic risk.

In conclusion, the present study provides biochemical evidence of enhanced in vivo lipid peroxidation in the most common genetic cause of severe hyperhomocysteinemia and identifies a novel mechanism through which elevated levels of this sulfur-containing amino acid could contribute to atherothrombosis.

Acknowledgments

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