Abnormally High Thromboxane Biosynthesis in Homozygous Homocystinuria
Evidence for Platelet Involvement and Probufol-sensitive Mechanism

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Abstract

Homocystinuria due to homzygous cystathionine β-synthase deficiency is an inborn error of metabolism characterized by a high incidence of thrombosis and premature atherosclerosis. We evaluated TXA2 biosynthesis in vivo and several in vitro tests of platelet function in 11 homocystinuric patients and 12 healthy controls. In vitro, patients’ platelet aggregation was within control values as were TXB2 formation, fibrinogen binding, and ATP secretion in response to thrombin. In contrast, the urinary excretion of 11-dehydro-TXB2, a major enzymatic derivative of TXA2, was > 2 SD of controls in all patients (1,724±828 pg/mg creatinine, mean±SD, in patients vs. 345±136 in controls, P < 0.001). The administration to four patients of low-dose aspirin (50 mg/d for 1 wk) reduced metabolite excretion by > 80%. The recovery of 11-dehydro-TXB2 excretion over the 10 d that followed aspirin cessation occurred with a pattern consistent with the entry into the circulation of platelets with intact cyclooxygenase activity. Prolonged partial reduction in the abnormally high excretion of both 11-dehydro-TXB2 and 2,3-dinor-TXB2, was also observed in seven patients who ingested 500 mg daily for 3 wk of the antioxidant drug probucol. These results provide evidence for enhanced thromboxane biosynthesis in homocystinuria and for its partial dependence on probucol-sensitive mechanisms. Furthermore, the elevated TXA2 formation in homocystinuria is likely to reflect, at least in part, in vivo platelet activation. (J. Clin. Invest. 1993, 92:1400–1406.) Key words: cystathionine β-synthase deficiency • 11-dehydro-TXB2 • 2,3-dinor-TXB2 • aspirin • antioxidant

Introduction

Homocystinuria due to cystathionine β-synthase deficiency (CBSD) is a recessively inherited error of metabolism strongly associated with premature atherosclerosis and arterial and venous thrombosis (1–4). Some hemostatic factors that may contribute to this tendency have been investigated in a large series of studies (5–13). Plasma coagulation factor VII, platelet survival, and antithrombin III activity have been reported to be abnormal (5–10). However, these claims have been challenged (11–13). Biochemical measurements of urinary TX metabolites and clinical trials with aspirin indicate that enhanced biosynthesis of TXA2 by platelets is an important contributor to the risk of thrombosis associated with several cardiovascular risk factors (14). Abnormally high levels of 2,3-dinor-TXB2, an enzymatic derivative of TXA2, have been reported in the urines of a CBSD patient (15). Therefore, we investigated TXA2 biosynthesis and its inhibition by low-dose aspirin, as reflected by the urinary excretion of its major metabolites, 11-dehydro-TXB2 and 2,3-dinor-TXB2, in a larger group of CBSD patients and controls with the aim of establishing the prevalence of this biochemical abnormality and its cellular source.

Hemostatic abnormalities involving antithrombin III, fibrinogen, plasminogen activator inhibitor-1, or tissue plasminogen activator have been reported in other clinical settings characterized by a thrombotic tendency (16–18). These abnormalities are currently thought to be associated with high circulating levels of thrombin. In addition to its role in the conversion of fibrinogen to fibrin, thrombin is a potent inducer of platelet TXA2 biosynthesis. Low levels of antithrombin III, the major plasma inhibitor of thrombin, have been reported in CBSD patients (6–8). To examine the possibility that the enhanced excretion of TXA2 metabolites in homocystinuria might be due to high circulating levels of thrombin, we measured several hemostatic variables that reflect a hypercoagulable state. The administration of probucol has been reported to prevent the increased TXA2 biosynthesis of cholesterol-fed rats (19). This has been suggested to depend on probucol-sensitive oxidative mechanisms (19). To gain insight into the mechanisms responsible for enhanced thromboxane biosynthesis in homocystinuria, the effects of a short-term course of probucol therapy on metabolite excretion were assessed in seven CBSD patients. Our results provide preliminary evidence that probucol may partially reduce the enhanced in vivo biosynthesis of TXA2 in homocystinuria.

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1. Abbreviation used in this paper: CBSD, Homocystinuria due to cystathionine β-synthase deficiency.
Methods

Subjects. 11 CBSD patients (5 females, 6 males, 8-39 yr) belonging to 8 unrelated families and 17 healthy control subjects (7 females, 10 males, 14-44 yr) were studied. None of the control subjects had a history of disease known to alter platelet function. All patients fulfilled the criteria (20) for the diagnosis of the homozygous form of CBSD in that all exhibited ectopia lentis, homocYSTINuria, and abnormally high plasma levels of methionine and homocysteine with low or undetectable levels of cystine (21).

All but one (see Table I) were responsive to pyridoxine and had been on pyridoxine treatment (600-900 mg/d) from the time of the diagnosis. The pyridoxine-unresponsive subject was on 8 g/d betaine. Skin fibroblast cultures (22) showed an abnormally low activity (< 1% of controls) of cystathionine θ-synthase in four patients. The other seven patients were not evaluated. For all the data reported in the results section, no statistically significant differences were found between the four patients tested and the other seven patients. All patients were comparable to controls with respect to renal function (patients vs. controls, mean±1 SD; serum creatinine concentration 0.77±0.2 vs. 0.8±0.5 mg/dl; urine creatinine concentration, 0.9±0.3 vs. 1.0±0.5 mg/dl; and blood urea nitrogen, 47±3 vs. 30±2 mg/dl in controls); cigarette smoking; systolic (133±12 vs. 147±29) and diastolic blood pressure (79±2 vs. 84±10 mmHg; controls vs. patients, respectively); plasma glucose (83±17 vs. 79±12 mg/dl) and cholesterol (150±14 vs. 171±35 mg/dl), and triglyceride levels (78±23 vs. 97±48 mg/dl) (patients vs. controls). No patient or control subject showed S-T depression or Q waves on ECG and all patients' clinical summaries were negative for angina pectoris or myocardial infarction. Also, all had normal peripheral pulses and were negative for bruises over the carotid vessels. Duplex scanner analysis confirmed the negativity with respect to carotid arteries, while it revealed early signs of atherosclerotic vascular disease of the iliac arteries in 4 of the 11 patients. The vascular studies of these patients have been reported in detail elsewhere (4).

For all the data reported in the results section, no statistically significant differences were found between these four patients and the other seven homozygous CBSD patients studied.

Material, pADP, and ATP sodium salts, Heps, human albumin, hirudin, bovine fibrinogen, and trisodium citrate were from Sigma Chemical Co. (St. Louis, MO). pADP, ATP, and hirudin were dissolved in distilled water, prepared in 1 M solutions and stored in small aliquots at -20°C. A 1 M stock solution of Heps was prepared in RPMI and stored at 4°C. Trisodium citrate (3.8%) was prepared in sterile, pyrogen-free water. Epinephrine (epinephrine hydrochloride, isotonic solution) was from Elkins-Sinn, Inc. (Cherry Hill, NJ). Thrombin was from Parke-Davis (Detroit, MI). It was dissolved in distilled water and stored in small aliquots at -70°C. Equine tendon collagen was from Hormon-Chemie (Munich, Germany) and arachidonate from Nu Check Prep, Inc. (Elysian, MN). Firefly luciferase and luciferin (Chromolume 395) was from Chrono-Log Corp. (Havertown, PA). Silicone oils (methyl silicone 1.0 DC 200 and hi-phenyl silicone 125 DC 550) were purchased from W. F. Nye, Specialty Lubricants (New Bedford, MA). Mixtures of DC 200 and DC 550 and solutions of Chronolume as well as of the sodium salt of arachidonic acid were prepared as previously described (23). Sep-Pak C18 cartridges were from Waters Associates (Milford, MA); 11-dehydro-1H-TXB2 (70-120 Ci/mmol) was purchased from Amersham (Milan, Italy); and authentic TXB2, 2,3-dinor-TXB2, and 11-dehydro-TXB2 were kindly provided by Dr. J. Pike (The Upjohn Co., Kalamazoo, MI). Iodogen (1,3,4,6-tetracloro-3,6-diphenylglycoluril) was from Pierce Eurochemie (Oud Beijerland, The Netherlands). Human melanoma tissue plasminogen activator was obtained from Dr. D. Collen, Centre for Thrombosis and Vascular Research (Leuven, Belgium). Human plasminogen, CnBr-digested human fibrinogen, plasminogen activator inhibitor-1-depleted plasma and the chromogenic substrate for the assay of the activity of plasminogen activator inhibitor-1 were from Ortho Diagnostics (Milan, Italy). The kit for the measurement of the coagulant activity of fibrinogen (Clauss method) was from General Diagnostics (Milan, Italy) whereas that for the functional determination of antithrombin III was from Boehringer-Biochemia-Robin (Milan, Italy).

Urinary 11-dehydro-TXB2 and 2,3-dinor-TXB2 assays. These measurements were carried out on 20-ml aliquots of urine adjusted to pH 4.0-4.5 with formic acid, run on Sep-Pak C18 cartridges, and eluted with ethyl acetate. The eluate was then chromatographed on silicic acid columns, eluted with benzene/ethyl acetate/methanol (60:40:30), and immunoreactive 11-dehydro-TXB2 was measured by a previously described RIA (24, 25). This assay has been validated for the measurement of metabolite levels in human urine by comparison with negative ion-chemical ionization gas-chromatography/mass spectrometry (24). Immunoreactive 2,3-dinor-TXB2 was measured by a previously validated RIA (26) after separating the metabolite from TXB2. This was achieved by subjecting the eluates prepared for 11-dehydro-TXB2 measurements to reverse-phase high pressure liquid chromatography (solvent system, acetonitrile/water/acetic acid [27:73:0.18]; flow rate, 0.5 ml/min). Urine creatinin was determined according to the manufacturer’s recommendations (Behring Testomar-Creatininina Combipack, Scoppito, Italy).

Studies on platelets and fibrinolysis. Aggregation of platelets in platelet-rich plasma, TXB2 synthesis, and ATP secretion in response to 1 or 5 U/ml thrombin, preparation of suspensions of washed platelets, purification and labeling of human fibrinogen, thrombin-dependent fibrinogen binding, and analysis of the binding data were performed as previously described (23). For studies on the sensitivity of platelets to aggregating agents, the AC50 was calculated. This was defined as the concentration of an agonist that produced 50% light transmittance within 3 min after addition to the platelet suspension. For binding studies, hirudin was added in microliter volumes at a final concentration of 0.1 U/ml to untrissted platelet suspensions to prevent clotting of fibrinogen by thrombin (0.1 U/ml). Fibrinolytic activity in the plasma euglobulin fraction was determined before and after 10 min of venous stasis and expressed in tissue plasminogen activator equivalent units with reference to data obtained using purified melanoma tissue plasminogen activator. Tissue plasminogen activator- and inhibitor-1-related antigens were measured by an ELISA method according to the manufacturer’s recommendations, whereas the biological activity of the plasminogen activator inhibitor was studied by an amidolytic assay (27).

Other assays. The total cholesterol, triglyceride, and plasma glucose levels were measured according to standard procedures (27).

Clinical protocols. Informed consent was obtained from all patients and volunteers after approval of the protocols by the Human Investigation Committee, and the studies were carried out according to the principles expressed in the Declaration of Helsinki. Because aspirin is present in so many over-the-counter preparations, all subjects were asked to avoid cold remedies during the study. Neither patients nor controls had taken any medication known to interfere with arachidonate metabolism or platelet function for ≥ 1 wk before blood sampling. On every occasion and for each subject, after 12–15 h of overnight fasting, 45 ml of blood was collected at 9–9.30 a.m., from the antecubital vein, via a 19-gauge scalp vein needle, into a sterile plastic tube containing 5 ml of sterile 3.8% trisodium citrate, and was immediately processed.

In the first phase of the study, a comparison of 11-dehydro-TXB2 excretion and in vitro platelet function between CBSD patients and controls was performed. For these determinations, blood samples collected in the morning as reported above, and the urines, collected during the overnight fasting, were used. The urines were immediately frozen and kept at -70°C until extracted. Nine CBSD patients were studied after being on long-term treatment with pyridoxine (Table I). Two newly diagnosed patients were studied both before and 4–12 wk after starting pyridoxine. To further investigate the potential effect of this therapy on 11-dehydro-TXB2 excretion, three healthy volunteers (all males, aged 26–30 yr) were instructed to take pyridoxine 900 mg/d for 4 wk and urines were collected twice before treatment and at 1, 2, 3, and 4 wk on treatment. Results from the first phase of the study indicated an abnormally high 11-dehydro-TXB2 excretion and a normal in...
vitro platelet function in CBSD. Therefore, we examined the relative contribution of platelets to the enhanced metabolite excretion and the hypothesis that a hypercoagulable state might be involved in this in vivo abnormality of arachidonate metabolism. To address the first issue, four CBSD patients were instructed to ingest 50 mg aspirin (Bayer Italia, Milan, Italy) daily for 1 wk with the evening meal. 12-h urines were collected before, on the 4th and 7th day of treatment, and on the 4th and 10th day after stopping aspirin. To address the second issue, peripheral venous blood was obtained from nine patients on two different occasions before the aspirin study, and plasma samples were immediately processed for the measurement of a series of hemostatic variables.

In the last phase of the study, 500-mg tablets of probucol (Lurselle; Lepetit, Milan, Italy) were administered daily for 3 wk to 7 patients, and 12-h urines were collected before, on the last day of treatment, and 3 wk later. To determine whether the inhibitory effect of probucol on 11-dehydro-TXB2 excretion was due to impaired TXA2 biosynthesis or to a shift in its metabolic disposition from the 11-hydroxydehydrogenase pathway (28), the urinary excretion of the metabolite generated through the β-oxidation pathway, 2,3-dinor-TXB2, was also measured. To interpret changes in 11-dehydro-TXB2 excretion associated with probucol administration to CBSD patients, 5 healthy subjects were instructed to take 500 mg probucol daily for 3 wk, and 12-h urines were collected before; at the end of the 1st, 2nd, and 3rd wk of treatment; and during the following 3 wk of wash out.

Statistical analysis. Results are expressed as means±1 SD. The distribution of the variables was analyzed by nonparametric comparisons, the variance by the Kruskall–Wallis method, and pairwise comparisons by the Mann–Whitney U test.

Results

Studies on the urinary excretion of 11-dehydro-TXB2. In control subjects the urinary excretion of 11-dehydro-TXB2 averaged 345±136 pg/mg creatinine (range, 138–606). CBSD patients had significantly (P < 0.001) higher metabolite excretion: 1,724±828 pg/mg creatinine. All patients had metabolite excretion > 2 SD above the control mean. Enhanced thromboxane biosynthesis was unrelated to cardiovascular risk factors and independent of detectable atherosclerotic vascular disease (Table II). 11-dehydro-TXB2 measurements were highly reproducible upon repeated sampling. Over a 1-yr period, three different collections from three CBSD patients showed that the intrasubject coefficient of variation did not exceed 16% (3,280, 3,201, and 3,351 pg/mg creatinine in the urines from S.S.; 2,173, 2,791, and 2,269 in those from S.R.; and 1,250, 1,308, and 956, in those from T.G.). Two newly diagnosed patients (P.V. and T.L., see Table I) provided an opportunity to assess the potential impact of pyridoxine treatment on urinary 11-dehydro-TXB2 excretion. Patient P.V. had a baseline excretion rate of 649 pg/mg creatinine and repeated measurements at 4 and 12 wk after starting therapy yielded comparable values of 696 and 547, respectively (intrasubject CV, 12%). Patient T.L. had two baseline measurements of 11-dehydro-TXB2 of 2,891 and 3,101 pg/mg creatinine and a third one while on pyridoxine treatment since 6 wk of 2,703 (intrasubject CV, 7%). To further document that this form of treatment is not responsible for the changes observed, 11-dehydro-TXB2 excretion was measured in three healthy subjects before and up to 4 wk after starting pyridoxine treatment. Metabolite excretion averaged 385±151 and 365±126 pg/mg creatinine on two separate occasions before treatment and remained relatively stable at 313±133, 350±138, 381±96, and 369±145 at 1, 2, 3, and 4 wk of pyridoxine 900 mg/d (intrasubject CV, 10.2±6.3%).

Effect of low-dose aspirin. To characterize the cellular origin of the enhanced biosynthesis of TXA2 in CBSD, we evaluated the short-term effects of a relatively platelet-selective regimen of aspirin (50 mg/d for 1 wk) in four patients (S.R., S.S., Q.E., and T.G.). Before the administration of aspirin, the rate of urinary 11-dehydro-TXB2 excretion averaged 1,977±1068 pg/mg creatinine. After 4 and 7 d of treatment, metabolite excretion was reduced by 82–84% (348±287 and 309±293 pg/mg, respectively, P < 0.001 vs. preaspirin values). After withdrawal of aspirin, the pattern of 11-dehydro-TXB2 excretion (Fig. 1) was consistent with a platelet origin of the parent compound, its recovery being compatible with the slow entry into

### Table I. Clinical Features of the CBSD Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Duration of treatment* yr</th>
<th>Age at diagnosis</th>
<th>B4 responsive</th>
<th>Cystathionine β-synthase activity %</th>
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<tbody>
<tr>
<td>SR</td>
<td>F</td>
<td>12</td>
<td>6</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>SS</td>
<td>F</td>
<td>12</td>
<td>2</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>DAM</td>
<td>M</td>
<td>12</td>
<td>10</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>DA</td>
<td>F</td>
<td>12</td>
<td>12</td>
<td>+</td>
<td>ND</td>
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<tr>
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<td>F</td>
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<td>9</td>
<td>+</td>
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<tr>
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<td>M</td>
<td>13</td>
<td>3</td>
<td>+</td>
<td>&lt; 1</td>
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<tr>
<td>QE</td>
<td>M</td>
<td>14</td>
<td>25</td>
<td>+</td>
<td>&lt; 1</td>
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<tr>
<td>IA</td>
<td>M</td>
<td>13</td>
<td>13</td>
<td>+</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>TG†</td>
<td>M</td>
<td>10</td>
<td>4</td>
<td>—</td>
<td>ND</td>
</tr>
<tr>
<td>PV</td>
<td>M</td>
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<td>28</td>
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<tr>
<td>TL</td>
<td>F</td>
<td>—</td>
<td>8</td>
<td>+</td>
<td>ND</td>
</tr>
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</table>

* Pyridoxine, 600–900 mg/d. † B4 nonresponsive, on treatment with betaine (8 g/d). ND, not determined.

### Table II. Urinary Excretion of 11-Dehydro-TXB2 in CBSD Patients, in Relation to Blood Lipids and Glucose and Presence of Vascular Disease

<table>
<thead>
<tr>
<th>Patient</th>
<th>Plasma Cholesterol mg/dl</th>
<th>Plasma TG mg/dl</th>
<th>Plasma Glucose mg/dl</th>
<th>Atherosclerotic vascular disease*</th>
<th>11-dehydro-TXB2 excretion† pg/mg creatinine</th>
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</thead>
<tbody>
<tr>
<td>SR</td>
<td>152</td>
<td>85</td>
<td>96</td>
<td>—</td>
<td>2,173</td>
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<tr>
<td>SS</td>
<td>170</td>
<td>63</td>
<td>75</td>
<td>+</td>
<td>3,280</td>
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<tr>
<td>DAM</td>
<td>141</td>
<td>100</td>
<td>103</td>
<td>+</td>
<td>1,312</td>
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<tr>
<td>DA</td>
<td>149</td>
<td>99</td>
<td>80</td>
<td>—</td>
<td>1,562</td>
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<tr>
<td>MA</td>
<td>157</td>
<td>86</td>
<td>109</td>
<td>+</td>
<td>1,234</td>
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<td>73</td>
<td>77</td>
<td>—</td>
<td>921</td>
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<td>62</td>
<td>86</td>
<td>—</td>
<td>2,062</td>
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<tr>
<td>TG</td>
<td>169</td>
<td>54</td>
<td>73</td>
<td>+</td>
<td>1,250</td>
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<td>69</td>
<td>—</td>
<td>649</td>
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<tr>
<td>TL</td>
<td>132</td>
<td>51</td>
<td>50</td>
<td>—</td>
<td>2,996</td>
</tr>
</tbody>
</table>

* Assessed by duplex scanner analysis and defined as > 15% narrowing of iliac arteries (4). † In 12 healthy subjects, 11-dehydro-TXB2 averaged 345±136 pg/mg creatinine (range: 138–606). TG, triglycerides.
the circulation of platelets with intact cyclooxygenase activity (29–33).

Studies on platelets, coagulation, and fibrinolysis. As shown in Table III, aggregation of platelets, as well as thrombin-dependent TxB2 synthesis, ATP secretion, and fibrinogen binding were comparable in CBSD patients and controls. Likewise, the coagulation and fibrinolytic factors analyzed were all within control values. These values are similar to those previously reported for normal subjects (16–18). No correlation was found between any of the parameters reported in this section and 11-dehydro-TXB2 excretion in CBSD.

Effect of probucol on 11-dehydro-TXB2 and 2,3-dinor-TXB2 excretion. To gain insight into the mechanism(s) responsible for enhanced TXA2 biosynthesis in CBSD, the antioxidant drug probucol was given daily for 21 d to seven patients (S.R., S.S., D.A.M., D.A., M.A., M.L., and T.G.). The rate of TX metabolite excretion at baseline averaged 1,647±981 pg/mg creatinine. At the end of probucol treatment (Fig. 2, top), 11-dehydro-TXB2 excretion was reduced by ~40% (P < 0.01 vs. preprobucol values). Plasma cholesterol was also significantly (P < 0.05) reduced from 154±12 to 131±9 mg/dl. 3 wk after withdrawing drug therapy, at a time when plasma cholesterol was comparable to baseline values (140±35, P < 0.05), 11-dehydro-TXB2 excretion was still significantly reduced (P < 0.005 vs. preprobucol data). We interpret this prolonged inhibition to reflect the very long half-life of the drug (34). The response to probucol showed a large interindividual variation in that the patients experienced a 30–70% reduction in metabolite excretion. The effect of probucol on TXA2 biosynthesis was further analyzed by measuring the urinary excretion of the metabolite generated through the β-oxidation pathway, 2,3-dinor-TXB2 (28).

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### Table III. Platelet, Coagulation, and Fibrinolytic Parameters in CBSD Patients and Controls

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control subjects</th>
<th>CBSD patients</th>
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<tbody>
<tr>
<td>Platelet aggregation (ACso)</td>
<td></td>
<td></td>
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<tr>
<td>ADP (μg/M)</td>
<td>1.8±0.6</td>
<td>1.3±0.3</td>
</tr>
<tr>
<td>Collagen (μg/ml)</td>
<td>0.6±0.2</td>
<td>0.4±0.2</td>
</tr>
<tr>
<td>Arachidonate (mM)</td>
<td>0.5±0.2</td>
<td>0.4±0.3</td>
</tr>
<tr>
<td>Epinephrine (μM)</td>
<td>5.1±0.3</td>
<td>4.6±1.5</td>
</tr>
<tr>
<td>Platelet ATP secretion (pmol/10^14 plts)</td>
<td>3.2±0.4</td>
<td>3.6±0.3</td>
</tr>
<tr>
<td>Platelet TXB2 (pmol/3 x 10^4 plts)</td>
<td>4.2±0.7</td>
<td>3.8±0.6</td>
</tr>
<tr>
<td>Platelet fibrinogen binding molecules/plt (×10^4)</td>
<td>6.0±0.8</td>
<td>6.3±0.6</td>
</tr>
<tr>
<td>K (μM/liter)</td>
<td>0.2±0.07</td>
<td>0.3±0.04</td>
</tr>
<tr>
<td>Plasma fibrinogen (mg/liter)</td>
<td>25.4±2.8</td>
<td>28.0±3.6</td>
</tr>
<tr>
<td>Antithrombin III (U/ml)</td>
<td>9.7±1.8</td>
<td>10.4±0.7</td>
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<tr>
<td>t-PA antigen (ng/ml)</td>
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<td></td>
</tr>
<tr>
<td>Pretrans</td>
<td>4.5±2.4</td>
<td>5.5±1.7</td>
</tr>
<tr>
<td>Posttrans</td>
<td>14.4±6.3</td>
<td>12.0±6.2</td>
</tr>
<tr>
<td>Fibrinolytic activity (U/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretrans</td>
<td>1.5±0.3</td>
<td>1.6±0.9</td>
</tr>
<tr>
<td>Posttrans</td>
<td>10.8±4.9</td>
<td>7.4±3.8</td>
</tr>
<tr>
<td>PAI-I antigen (ng/ml)</td>
<td>13.9±4.3</td>
<td>17.6±4.7</td>
</tr>
<tr>
<td>PAI activity (U/ml)</td>
<td>8.0±2.6</td>
<td>9.8±2.5</td>
</tr>
</tbody>
</table>

Each value is the mean±1 SD of the data obtained in 9 CBSD patients and 10 healthy control subjects. P > 0.05 for all comparisons between patients and controls. plt, platelet; t-PA: tissue plasminogen activator; PAI: plasminogen activator inhibitor.

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**Figure 1.** Urinary excretion of 11-dehydro-TXB2 during and after 1-wk administration of low-dose aspirin (50 mg/d) to four CBSD patients. The excretion of the metabolite is expressed as percentage (mean±SD) of pre-aspirin levels. Each patient served as her/his own control.

**Figure 2.** Urinary excretion of 11-dehydro-TXB2 (top) and 2,3-dinor-TXB2 (bottom) during and after 3-wk administration of probucol (500 mg/d) to seven CBSD patients. Mean±1 SD values are represented. For both metabolites, P < 0.005 time 0 vs. 6-wk values. For 11-dehydro-TXB2, P < 0.01 time 0 vs. 3-wk or 3- vs. 6-wk values.
reduction in urinary excretion of 11-dehydro-TXB₂ and that of
2,3-dinor-TXB₂ (Fig. 3).
At variance with the effect on TXB₂ metabolite excretion
observed in CBSD, the same regimen of probucol did not affect
11-dehydro-TXB₂ excretion in five control subjects to any sta-
tistically significant extent. Thus, metabolite excretion aver-
aged 360±115 pg/mg creatinine at baseline; 368±86, 320±116, and
392±124 at the end of the 1st, 2nd, and 3rd wk of probucol ad-
ministration; respectively, and remained stable at 388±108, 373±94, and
401±115 pg/mg creatinine over the following 3 wk after drug withdrawal.

Discussion
In its homozygous form, homocystinuria due to cystathionine
β-synthase deficiency is characterized by multiple biochemical
abnormalities, mental retardation, ectopia lentis, osteoporosis,
premature atherosclerosis, and venous and arterial thrombosis.
Although the mechanism is largely unknown, the adminis-
tration of pharmacological doses of vitamin B₆ (100–1,000 mg/d)
appears to correct, at least in part, most clinical symptoms and
biochemical abnormalities in about half of the patients. In con-
trast, the problem of premature atherosclerosis and its throm-
botic complications continues to be an important one in the
management of CBSD (1–4). At variance with the homozy-
gous state, heterozygosity for homocystinuria is a rather com-
mon entity (1 of 100–200 live births) and recent reports (3, 4,
35) indicate that the homocysteinuric trait is associated with a
similar tendency to atherosclerosis and its thrombotic compi-
lations. The pathophysiological and clinical implications of
this disease, as well as the observation that abnormalities in
platelet arachidonate metabolism are often present in patients
at risk for thrombosis (14), prompted us to study thromboxane
biosynthesis in CBSD. The urinary excretion of 11-dehydro-
TXB₂, a major enzymatic derivative of TXA₂, was approxi-
mately fivefold higher in CBSD patients than controls. This
alteration was independent of the presence of other cardiovas-
cular risk factors or atherosclerotic vascular disease. Moreover,
the finding of similarly increased excretion of 2,3-dinor-TXB₂
strongly argues for these biochemical changes reflecting en-
hanced biosynthesis of TXA₂ rather than altered metabolic dis-
position in the setting of CBSD.
Urinary eicosanoid metabolites do not reflect a specific site
of eicosanoid biosynthesis (14). In an attempt to characterize
the cellular source of the abnormally high TXA₂ biosynthesis
in homocystinuria, we administered aspirin, 50 mg daily for 1
wk, and evaluated the pattern of inhibition and recovery of
11-dehydro-TXB₂ excretion in four patients. This regimen
is known to impair platelet TXA₂ synthesis in a relatively selec-
tive cumulative fashion (30, 31). The finding of 82–84% sup-
pression of TX metabolite excretion upon repeated daily dos-
ing with 50 mg of aspirin suggests that this was achieved
through a process of cumulative inhibition, inasmuch as a sin-
gle administration of the same dose only produces 50% inhibi-
tion of platelet cyclooxygenase activity (30). A pattern of cu-
mulative inhibition is consistent with the rate of enzyme turn
over or cell renewal being longer than the 24-h dosing interval.
Both such profound suppression and the incomplete recovery
of TX metabolite excretion at 4 and 7 d after aspirin with-
drawal are compatible with this pattern, reflecting cumulative
acetylation by aspirin of platelet prostaglandin/H-synthase and
the slow entry of new platelets with intact enzyme into the
systemic circulation, respectively. However, these consider-
ations do not exclude the possibility that extraplatelet sources
(e.g., macrophages infiltrating atherosclerotic lesions) of TXA₂
biosynthesis may contribute, at least in part, to enhanced me-
tabolite excretion in the setting of homozygous homocysti-
uria.
In type II diabetes mellitus, enhanced TX biosynthesis is
associated with increased in vitro sensitivity of platelets to ag-
gragating agents (36). Therefore, we examined platelet func-
tion ex vivo. The data obtained indicate that platelets from
CBSD patients aggregate, secrete ATP, form TXB₂, and bind
fibrinogen in a fashion indistinguishable from controls.
Changes in the plasma levels of some hemostatic variables are
thought to be associated with high circulating levels of throm-
bin (15–18). To address the possibility that thrombin might be
involved in the abnormally high TXA₂ biosynthesis in homocys-
tinuria, we measured several hemostatic indexes of hyper-
coagulability. The results obtained argue against the hypothe-
sis of an hypercoagulable state being responsible for platelet acti-
vation resulting in enhanced TXA₂ biosynthesis.
Kaplan et al. (19) have shown that the antioxidant drug
probucol prevented the hemodynamic abnormalities as well as
the increased TXA₂ biosynthesis in rats on short-term chole-
terol feeding. To gain insight into the mechanism(s) responsi-
bile for enhanced TXA₂ biosynthesis in homocystinuria, we
evaluated the effects of a 3-wk administration of probucol on
the urinary excretion of 11-dehydro-TXB₂ and 2,3-dinor-
TXB₂. Both were partially, though persistently, reduced by this
administration, suggesting an effect of probucol on TXA₂ biosyn-
thesis. This is unlikely to reflect a direct inhibitory effect of pro-
abul on platelet function or enzymes involved in arachidonate
metabolism, inasmuch as 11-dehydro-TXB₂ excretion was un-
altered in control subjects exposed to the same drug regimen.
The study of Kaplan et al. (19) showed that although the ad-
ministration of probucol did not affect the feeding-related rise
in low density lipoproteins, and only partially prevented that
of total cholesterol, it entirely corrected the increased TXB₂ ex-
creation of cholesterol-fed rats. Cholesterol levels were entirely
normal in our patients, and no correlation was found between
baseline levels of plasma cholesterol and urinary 11-dehydro-TXB₂ (Table II). 3 wk after withdrawing probucol, the urinary excretion of TX metabolites was still significantly reduced, at a time when plasma cholesterol was not significantly different from preprobucol values.

Thus, although elevated levels of plasma cholesterol may be causally related to elevated 11-dehydro-TXB₂ excretion in type IIa hypercholesterolemia (37), the rather trivial (9–15%) reduction in blood cholesterol vis-a-vis the 40–60% drop in TX metabolite excretion, the lack of correlation between the two, and the time-dependent dissociation of the observed changes during and after probucol treatment, all argue against the involvement of cholesterol in the enhanced TXA₂ biosynthesis in homocystinuria as well as in its partial correction by probucol. The production of oxidized low density lipoproteins is facilitated by thiol compounds, including homocysteine (38, 39). Oxidized low density lipoproteins induce platelet activation and TX formation (40). In vitro incubation of platelet-rich plasma with homocysteine increases the formation of TXB₂ by platelets (41). In vitro, probucol prevents the oxidative modification of low density lipoproteins (34, 42). Thus, in spite of the limitations inherent to a short-term, open study, inhibition by probucol of the elevated TXA₂ biosynthesis is consistent with the possibility that in vivo oxidized low density lipoproteins mediate, at least in part, the increased platelet arachidonic metabolism in homocystinuria.

It has also been suggested that probucol’s antiatherosclerotic activity may be related to inhibition of interleukin 1 secretion by macrophages (43, 44). Thus, an effect of probucol on extraplatelet sources of TXA₂ biosynthesis, independent of its antioxidant activity, cannot be excluded by our findings. The possibility of an important role of macrophages would be consistent with unaltered platelet TXA₂ production in vitro and enhanced TXA₂ biosynthesis in vivo, as detected in our CBSD patients. The use of MDL 29,311, which is an analogue of probucol that does not lower cholesterol but does have full antioxidant activity (45), or other antioxidant interventions might help to clarify these mechanistic uncertainties.

These results provide biochemical evidence of enhanced TXA₂ biosynthesis in homocystinuria and of its partial dependence on probucol-sensitive mechanisms. Our data also suggest that this abnormality may reflect the activation of platelets and other cells with comparable aspirin sensitivity in response to stimuli operating in vivo, the nature of which remains to be determined. We believe that information from these studies allows for a better understanding of the tendency to thrombosis in homocystinuria and helps identify the mechanism(s) of homocysteinemia as a risk factor for atherosclerotic cardiovascular disease. Finally, our results provide a rationale for testing the efficacy and safety of low-dose aspirin in this setting.

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