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Effect of Homocysteine and Homocystine on Platelet and Vascular Arachidonic Acid Metabolism

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Summary

Normal hemostasis depends in part on the balance achieved between proaggregatory and prothrombotic platelet thromboxane A_2, measured as its stable end-product thromboxane B_2 (TXB_2), and vascular prostacyclin (PGI_2), which inhibits platelet aggregation and is antithrombotic. Cystathionine-β-synthase deficiency is characterized by a high frequency of thromboembolic disease. We therefore studied, *in vitro*, the effects of homocysteine and related compounds on platelet TXB_2 and vascular PGI_2 formation.

In paired samples of platelet rich plasma, which had been preincubated with L-homocystine (1 mM), mean production of the two platelet cyclooxygenase products, TXB_2 and 12-hydroxy-5,8,10-heptadecatrienoic acid increased significantly from control levels [13.6% ± 1.9 to 19.8% ± 2.1 ($P < 0.02$) TXB_2 and 29.8% ± 4.2 to 39.4% ± 4.1 ($P < 0.01$) HHT]. In the presence of D,L-homocysteine (1 mM), mean platelet TXB_2 and 12-hydroxy-5,8,10-heptadecatrienoic acid production was also significantly increased [12.7% ± 1.5 to 16.9% ± 1.5 ($P < 0.01$) TXB_2 and 27% ± 4 to 31% ± 4.1 ($P < 0.02$) HHT]. Cystine, cysteine, or methionine (1 mM) did not have similar effects in this test system. Homocysteine and homocystine were without effect on the synthesis of vascular PGI_2 by umbilical artery segments [control, 0.22 ± 0.03 to 0.21 ± 0.03 ng/mg with D,L-homocysteine and 0.20 ± 0.04 control to 0.19 ± 0.04 ng/mg with D,L-homocystine]. A homocyst(e)ine-induced increase in platelet thromboxane production in the absence of an increase in vascular prostacyclin, if present *in vivo*, may contribute to the vascular thromboses characteristic of human homocystinemias (homocystinurias).

The homocystinurias occur as a result of genetically determined defects in the metabolism of homocysteine (15). The most common etiology, cystathionine-β-synthase deficiency, results in a decrease in the rate of conversion of homocysteine to cystathionine and is generally accompanied by hypermethioninemia. Deficiencies in the remethylation of homocysteine to methionine cause homocystinemia accompanied by hypomethioninemia but are much less common. In all these disorders, accumulated homocysteine is oxidized to homocystine, which is found in excessive amounts in blood and urine.

Cystathionine-β-synthase dificiency is characterized by ectopia lentis, skeletal deformities, central nervous system abnormalities and a high frequency of thromboembolic disease. Atherosclerosis and occlusion of major vessels such as myocardial, cerebral, renal and pulmonary arteries and veins may occur as early as the first decade, often with fatal results (15).

Studies on other disorders with a high incidence of thrombotic complications suggest that normal hemostasis depends in part on the balance achieved between proaggregatory and prothrombotic platelet thromboxane A_2 and vascular prostacyclin, which inhibits platelet aggregation and is thus antithrombotic (14). We therefore undertook an *in vitro* study of the effects of homocysteine and related compounds on platelet thromboxane and vascular prostacyclin formation.

MATERIALS AND METHODS

Evaluation of platelet arachidonic acid metabolism after incubation in vitro with homocystine, homocysteine, cystine, cysteine, or methionine. Blood samples were obtained after informed consent from control subjects using a two-syringe technique and 9 volumes of blood to 1 volume citrate-phosphate-dextrose solution. Platelet-rich plasma (PRP) was obtained by centrifugation of the samples at 200 × *g* for 20 min. PRP from each control was divided into three aliquots. Hank's balanced salt solution (HBSS) was added to one aliquot as a control, whereas L-homocystine or D,L-homocysteine (1 mM final concentration in HBSS) was added to each of the other two platelet aliquots. All samples were incubated at 37°C, at pH 7.4 for 45 min. Each patient's platelets served as their own control in order to evaluate the effects of the test compounds. In a second set of experiments, PRP from controls was divided into four aliquots and incubated with HBSS alone, or L-cystine, D,L-cysteine, or L-methionine (1 mM in HBSS) as described above.

To assess platelet conversion of [^{14}C]-arachidonic acid to metabolites by the lipoxygenase and cyclooxygenase enzymes, the platelets were washed (16) after the period of incubation, and resuspended in HBSS (37°C) containing 0.5 mM calcium chloride at a concentration of 5×10^8 platelets per ml. [^{14}C]-Arachidonic acid (specific activity 56.5 mCi/mM) made up as a sodium salt in 0.01 M Tris buffer, pH 7.4, was then added to a 1 ml platelet suspension (final concentration of arachidonate per sample was 6 μM). Aggregation was monitored in a Payton dual channel aggregometer. After 6 min, the samples were added to extraction vials containing 10 ml of absolute ethanol, then diluted, acidified with 1 N HCl to a pH of 3.3 and extracted into diethyl ether. Separation of [^{14}C]-arachidonic acid from thromboxane B_2 (TXB_2) was performed by thin layer chromatography of the free acids on silica gel G with diethyl ether:methanol:acetic acid (135:5:3, v/v) as eluting solvent. Thromboxane B_2 (TXB_2) standard (kindly supplied by Dr. John E. Pike, Upjohn Co., Kalamazoo, MI) was also applied to each plate. The plates were then scanned on a thin-layer radiochromatogram scanner (Berthold, Wildbad, W. Germany) and the silica gel corresponding to the TXB_2 peak scraped from the glass and counted in a scintillation counter. The remaining arachidonate and metabolites on the silica gel plates were extracted into 2 ml ether, then methylated using diazomethane, and separated on a thin layer chromatogram (silica gel B) using the organic layer of isooctane:water:ethyl acetate (2:2:1) as mobile

phase (5). The thin layer plates were scanned using a Berthold radiochromatogram scanner to determine the location of individual peaks. The thin layer plates were also subjected to autoradiography to improve the definition of 12-hydroxy-5,8, 10-heptadecatrienoic acid (HHT) and 12-hydroxy-5,8,10,14-eicosatetraenoic acid (HETE) as depicted in Figure 1. Areas corresponding to individual peaks were then scraped from the plate and counted in a Beckman liquid scintillation counter.

Statistical evaluation was performed using Student's t test for paired samples.

Evaluation of vascular prostaglandin I_2 by bioassay after incubation in vitro with homocystine and homocysteine. Umbilical cords were obtained from 8 normal full term vaginal deliveries in which there was no evidence for maternal ingestion of aspirin nor other drugs within two wk of delivery. The umbilical cords were dissected, and the umbilical arteries isolated and immediately cleaned externally in Ca^{++}- and Mg^{++}-free HBSS, pH 7.4, at 4°C. The arteries were cut into small rings. After weighing, approximately 6 arterial rings (~40 mg per tube) were suspended in HBSS (with 0.5 mM Ca^{++} and Mg^{++}) or HBSS (0.5 mM Ca^{++} and Mg^{++}) containing 1 mM L-homocystine or D,L-homocysteine, pH 7.4, in 1 ml final volumes. Following a 20-min incubation period, the vessels were washed in 1 ml HBSS, pH 7.4 at 22°C and resuspended in 120 μliters HBSS, pH 8.4, for 3 min at 22°C. Aliquots (1–120 μliters) of the supernatant were then added to control PRP 1 min before the addition of adenosine diphosphate (ADP) (3 μM final concentration). Prostacyclin activity was evaluated in a Payton dual channel aggregometer as described by Moncada *et al.* (13). The amount of antiaggregatory activity of both the control and test vascular rings was expressed as ng of PGI_2 per mg wet weight of tissue by extrapolation from a dose response standard curve obtained concomitantly with authentic PGI_2 (a gift from Dr. John E. Pike, Upjohn Co., Kalamazoo, MI). PGI_2 activity was further characterizied according to previous criteria (13). The activity was heat labile (lost by boiling for 0.25 min), unstable at room temperature (22°C for 20 min), and its generation was completely inhibited by 30-min preincubations with acetylsalicylic acid (100 μM) or indomethacin (5 μg/ml). Neutralization of prostacyclin activity was possible with an antiserum (kindly supplied by Dr. B. J. Smith, Philadelphia, PA), which selectively antagonized the activity of authentic PGI_2. A total of eight separate bioassay experiments were performed and the results evaluated using the t test for paired samples.

RESULTS

Effect of test compounds on platelet arachidonic acid metabolism. L-homocystine and D,L-homocysteine had significant effects on platelet arachidonic acid metabolism. Figure 2 depicts the effect of 1 mM homocystine on the metabolism of arachidonic acid through the cyclooxygenase pathway in six paired experiments. A significant increase in both TXB_2 and HHT was observed. Mean TXB_2 production increased from 13.6% ± 1.9 (1 S.E.) in control platelets to 19.8% ± 2.1 in paired samples, which had been preincubated with homocystine ($P < 0.02$). Mean HHT production increased from 29.8% ± 4.3 to 39.4% ± 4.1 ($P < 0.01$). Increases in TXB_2 and HHT production ranged from 18–140% in each of the six experiments. When the lipoxygenase product HETE was measured in the presence or absence of L-homocystine, no significant mean differences were observed (38.3% ± 4.2 and 41% ± 4.9, respectively).

As shown in Figure 3 similar results were observed in eight paired experiments in the presence of 1 mM D,L-homocysteine. Mean TXB_2 production was 12.7% ± 1.5 in control platelets, whereas paired samples in the presence of D,L-homocysteine averaged 16.9% ± 1.5 ($P < 0.01$). TXB_2 production increased from 13% to 85% over control values in six of eight paired experiments with two remaining relatively unchanged. HHT production was also increased from a mean control value of 27% ± 4 to 31% ± 4.1 ($P < 0.02$). HHT production increased from 14% to 104% over baseline control values in five of the eight paired experiments with three remaining relatively unchanged. When the lipoxygenase product HETE was measured in the presence or absence of D,L-homocysteine, mean values were similar (41.9% ± 3.2 and 41.5% ± 3.6, respectively).

In contrast to the above results, L-cystine, D,L-cysteine, or L-methionine had no effect on platelet metabolism through the cyclooxygenase (TXB_2 and HHT) or lipoxygenase (HETE) pathways (Table 1).

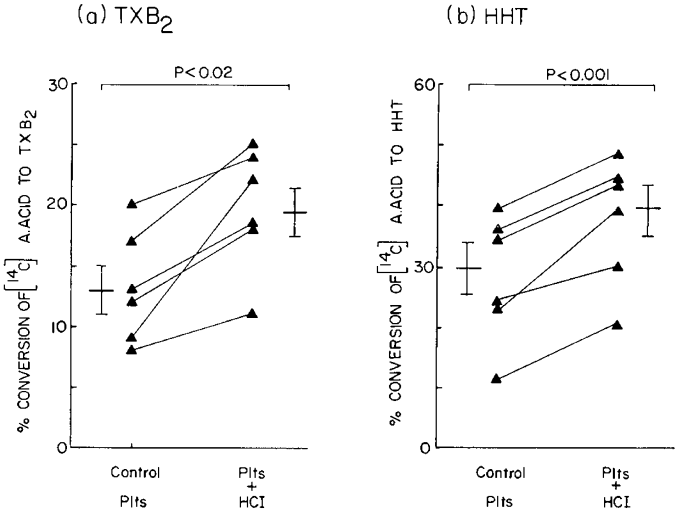

Fig. 2. Effect of 1 mM L-homocystine (HCl) on platelet thromboxane B_2 and HHT (12-hydroxy-5,8,10-heptadecatrienoic acid) formation in six paired experiments. Brackets show mean ± 1 SE.

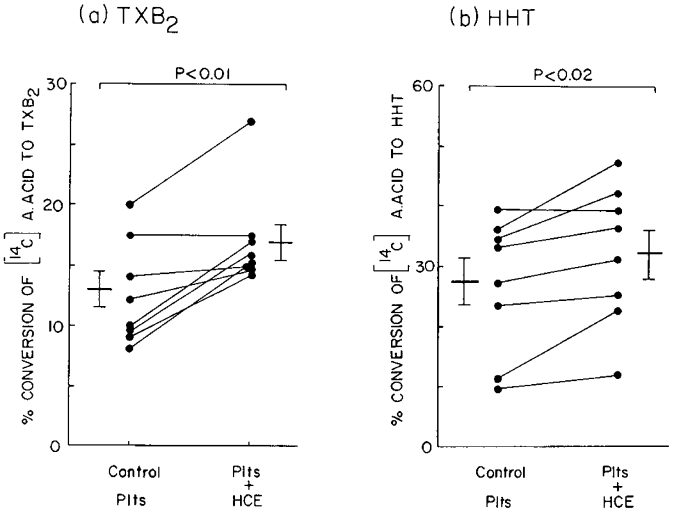

Fig. 3. Effect of 1 mM D,L-homocysteine (HCE) on platelet thromboxane B_2 and HHT (12-hydroxy-5,8,10-heptadecatrienoic acid) formation in eight paired experiments. Brackets show mean ± 1 SE.

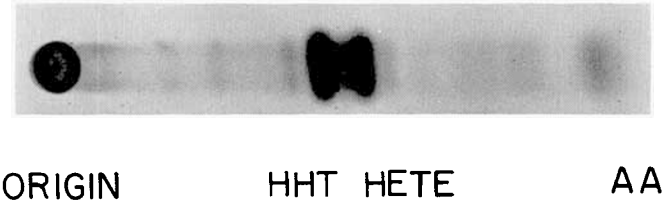

Fig. 1. Autoradiograph of a thin layer chromatogram of a platelet extract showing the separation of HHT (12-hydroxy-5,8,10-heptadecatrienoic acid) and HETE (12-hydroxy-5,8,10,14-eicosatetraenoic acid).

Table 1. *Effect of* L-*cystine,* D,L-*cysteine, and* L-*methionine on platelet thromboxane B_2, HHT and HETE formation in paired experiments*

Test Compound	TXB_2		HHT		HETE	
	Control	Expt.	Control	Expt.	Control	Expt.
L-cystine ($n = 6$)	12.5 ± 1.3[1]	11.2 ± 1.2	29.2 ± 2.5	28.1 ± 2.2	31.9 ± 2.1	32.8 ± 1.7
D,L-cysteine ($n = 6$)	15.6 ± 2.6	15.0 ± 2.0	30.3 ± 2.6	29.9 ± 2.8	33.3 ± 2.0	35.3 ± 2.8
L-methionine ($n = 7$)	12.3 ± 1.3	12.0 ± 1.6	28.4 ± 1.4	27.3 ± 0.9	34.1 ± 2.3	35.5 ± 2.4

[1] Mean (%) ± S.E., all paired mean differences are not significant ($P > 0.05$)

Effect of homocystine and homocysteine on prostaglandin I_2 production. As shown in Figure 4, no differences in bioassayable prostacyclin (PGI_2) production were observed between control vessels and paired samples incubated with either D,L-homocysteine or L-homocystine. Mean PGI_2 production was 0.21 ± 0.03 ng/mg vascular tissue in the presence of D,L-homocysteine compared to 0.22 ± 0.03 in paired control vessels; and 0.20 ± 0.04 ng/mg in control vessels paired to those preincubated with D,L-homocystine (0.19 ± 0.04).

DISCUSSION

Vascular occlusive lesions may lead to serious complications in patients with homocystinuria (15). Blood vessel alterations are similar to those of classic atherosclerosis, and are characterized by intimal thickening, medial disorganization and fibrosis with loss of elastic tissue, hyperplasia of smooth muscle cells and mural or occlusive thrombi (11). The precise etiology of these lesions is unknown. Our *in vitro* data suggest that in the presence of 1 mM homocystine or homocysteine, abnormalities in platelet arachidonic acid metabolism expected to favor thrombosis do occur.

In the platelet, arachidonic acid is liberated from cell membrane phospholipids after stimulation. Released arachidonic acid may then be converted by either the lipoxygenase or the cyclooxygenase pathway to various end products (Fig. 5). The lipoxygenase pathway converts arachidonic acid to 12L-hydroperoxy-5,8,10,14-eicosatetraenoic acid (HPETE) that is then reduced to HETE, a 12-hydroxy fatty acid. In contrast, the enzyme cyclooxygenase catalyzes the conversion of arachidonic acid to the endoperoxides PGG_2 and PGH_2. Thromboxane A_2 (TXA_2,) the most potent platelet aggregator and blood vessel constrictor known, is formed enzymatically from either PGG_2 or PGH_2 in the presence of thromboxane synthetase. TXA_2 is very unstable and undergoes rapid hydrolysis to its inactive end product TXB_2. The major alternative pathway for metabolism of the 20 carbon endoperoxides PGG_2 and PGH_2 in the platelet is to the 17 carbon hydroxy fatty acid, HHT and the 3 carbon fragment, malondialdehyde.

In contradistinction to these platelet products, arachidonic acid released from the endothelial lining of the vessel wall is converted to prostacyclin, also known as PGI_2, in the presence of vascular cyclooxygenase. PGI_2 inhibits platelet aggregation and causes vasodilatation. The balance between proaggregatory and prothrombotic platelet TXA_2 and antithrombotic prostacyclin appears to affect normal hemostasis. Imbalances in their production may lead to a thrombotic tendency (14).

In our paired *in vitro* studies, we have found significant increases in the production by platelets of the potent proaggregatory substance TXA_2 in the presence of L-homocystine and D,L-homocysteine. Because platelet HHT production is concomitantly elevated, these effects most likely occur as a result of increased activity of the platelet enzyme cyclooxygenase, rather than thromboxane synthetase (Fig. 1). At comparable concentrations, L-cystine, D,L-cysteine and L-methionine had no significant effects in this system. No changes in antiaggregatory vascular PGI_2 production were evident in our vascular incubation studies; thus, an imbalance in platelet-vascular prostaglandin synthesis favoring the production

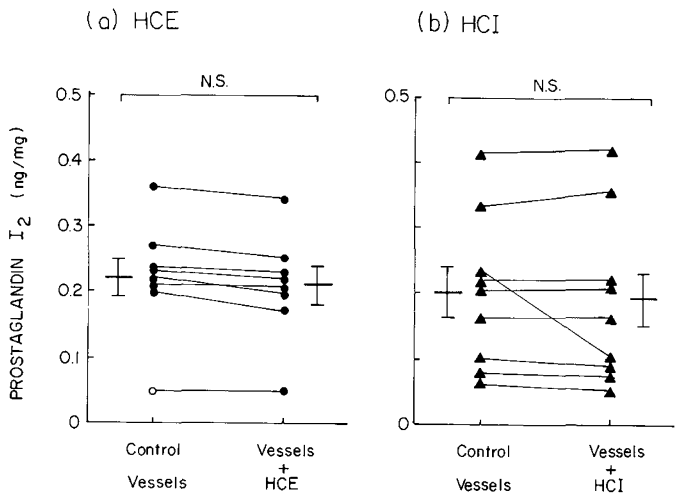

Fig. 4. Effect of D,L-homocysteine (HCE) and L-homocystine (HCI) (1 mM) on vascular prostaglandin (PGI_2) formation.

of prothrombotic platelet TXA_2 has been demonstrated *in vitro* in the presence of L-homocystine or D,L-homocysteine. Levels of 0.2 mM homocystine have been reported in patients with homocystinuria (15). Preliminary studies using *in vitro* concentrations of 0.2 mM gave equivocal results. Although an increase in both TXB_2 and HHT were observed in some experiments, the results were not statistically significant. We chose a higher concentration of 1 mM for all test compounds studied because unlike the *in vivo* situation, our *in vitro* experiments were conducted using necessarily limited periods of exposure.

Patients with cystathionine-β-synthase deficiency have elevated methionine levels. There is clinical evidence that methionine accumulation is unlikely to be important in the genesis of the atherothrombotic lesions. Several patients with homocystinemia and low or normal methionine levels have had vascular lesions very similar to those found in patients with homocystinemia and hypermethioninemia (2, 9, 10, 11). As reported, we were not able to obtain an effect on prostaglandin production *in vitro* using methionine.

The atherothrombotic complications in cystathionine-β-synthase deficiency are not considered to be adequately explained either by a structural abnormality in collagen (4) or by abnormalities in the fluid phase of coagulation (8). Platelet function or survival studies in homocystinemic humans have not revealed consistent abnormalities (1, 3, 7, 12, 17).

Platelet survival was decreased in L-homocysteine-infused primates compared to controls and these values returned toward normal upon treatment with the antiplatelet agent dipyridamole (6, 7). Patchy endothelial cell loss was seen in the animals chronically infused with homocysteine. These animals developed fibromusculoelastic lesions similar to those of atherosclerosis with the extent of lesions significantly decreased in the dipyridamole-treated group. The authors concluded that the underlying cause of the thrombotic and atherosclerotic tendency in homocystinemia

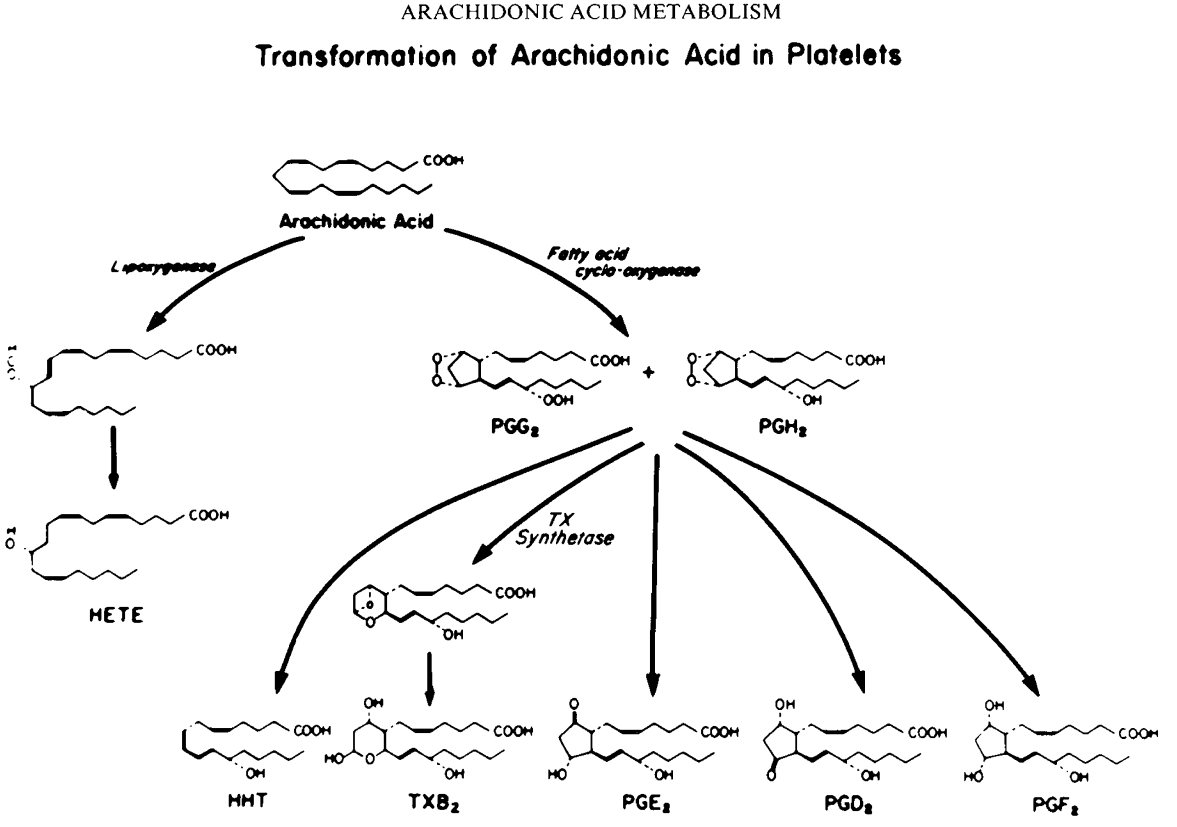

Fig. 5. Metabolism of arachidonic acid through the cyclooxygenase and lipoxygenase pathways in the platelet.

may be sustained homocyst(e)ine-induced vascular endothelial injury, with the formation of platelet thrombi on altered nonendothelialized surfaces as a secondary event; local release of platelet constituents, including a platelet mitogenic factor, was thought to mediate intimal proliferation of smooth muscle cells and play an important role in the genesis of the characteristic vascular lesions.

Our finding of increased platelet proaggregatory thromboxane, induced by homocysteine or homocystine in the absence of a compensatory increase in antithrombotic vascular PGI_2, appears compatible with the prothrombotic tendency seen in human homocystinemia. We recognize that these effects have been documented so far only under acute conditions *in vitro* and at concentrations of homocyst(e)ine in excess of the plasma concentrations chronically present in patients with cystathionine β-synthase deficiency (15). The possibility that a homocyst(e)ine induced proaggregatory increase in thromboxane production may contribute to the vascular occlusions in human homocystinemias is clearly not incompatible with the view that homocystinemia also induces vessel endothelial damage; both mechanisms, and/or others, could play a role in homocystinemic thromboembolism.

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