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HOMOCYSTEINE METABOLISM

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ABSTRACT

Homocysteine is a sulfur amino acid whose metabolism stands at the intersection of two pathways: remethylation to methionine, which requires folate and vitamin B_{12} (or betaine in an alternative reaction); and transsulfuration to cystathionine, which requires pyridoxal-5′-phosphate. The two pathways are coordinated by *S*-adenosylmethionine, which acts as an allosteric inhibitor of the methylenetetrahydrofolate reductase reaction and as an activator of cystathionine β-synthase. Hyperhomocysteinemia, a condition that recent epidemiological studies have shown to be associated with increased risk of vascular disease, arises from disrupted homocysteine metabolism. Severe hyperhomocysteinemia is due to rare genetic defects resulting in deficiencies in cystathionine beta synthase, methylenetetrahydrofolate reductase, or in enzymes involved in methyl-B_{12} synthesis and homocysteine methylation. Mild hyperhomocysteinemia seen in fasting conditions is due to mild impairment in the methylation pathway (i.e. folate or B_{12} deficiencies or methylenetetrahydrofolate reductase thermolability). Post–methionine-load hyperhomocysteinemia may be due to heterozygous cystathionine β-synthase defect or B_6 deficiency. Early studies with nonphysiological high homocysteine levels showed a variety of deleterious effects on endothelial or smooth muscle cells in culture. More recent studies with human beings and animals with mild hyperhomocysteinemia provided encouraging results in the attempt to understand the mechanism that underlies this relationship between mild elevations of plasma homocysteine and vascular disease. The studies with animal models indicated the possibility that the effect of elevated homocysteine is multifactorial, affecting both the vascular wall structure and the blood coagulation system.

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INTRODUCTION

Almost 30 years ago McCully reported that a child suffering from homocystinuria, cystathionuria, and methylmalonic aciduria, secondary to an abnormality of cobalamin metabolism, exhibited arterial lesions that were strikingly similar to those seen in patients with cystathionine β-synthase deficiency (88). This observation led to the proposal that the markedly elevated plasma homocysteine concentrations found in persons with homocystinuria were responsible for the development of premature occlusive vascular disease. In 1976, Wilcken & Wilcken published the first study showing that the homocysteine-cysteine mixed disulfide after a methionine load was significantly higher in patients with coronary artery disease (CAD) than in respective control subjects (150). The importance of their study lies in the fact that the levels of the mixed disulfide seen in these CAD patients, though higher than in healthy subjects, were nevertheless one order of magnitude lower than those seen in homocystinuric patients with congenital defects in cobalamin metabolism or cystathionine β-synthase deficiency. This landmark finding provided the basis for subsequent studies, which since 1990 have increased exponentially (112), and culminated in a meta-analysis published in 1995 (12). This meta-analysis identified a total of 27 studies involving more than 4000 patients with occlusive (cardiovascular, peripheral, and cerebrovascular) vascular disease and the same number of respective control subjects. The data synthesis showed that homocysteine was an independent, graded risk factor for atherosclerotic disease in the coronary, cerebral, and peripheral vessels. A 5-μM increment in total homocysteine (tHcy) plasma level is associated with an increased risk of 60% for men and 80% for women, of coronary heart disease.

A second review (112) counted a total of 42 additional studies that included ecological cardiovascular disease (CVD) mortality in 11 countries, cross-sectional, case control, nested case control, and cohort populations (112). Only six of these additional studies, including one prospective study, showed no association between homocysteine and disease or mortality. The rest showed positive associations with disease. Another review also suggested that mild hyperhomocysteinemia is associated with an increased risk of thrombotic disease (28). One of the most important studies involved patients with confirmed CAD in which plasma tHcy measured prospectively with a mean follow-up time of 4.6 years (106). After this period, only 3.6% of those with a tHcy level $<9.0\ \mu$M died, whereas in patients with a tHcy $>15\mu$M, the mortality was 24.7%. When a tHcy level below 9 μM was used as a reference, mortality rate increased 1.9-, 2.8-, and 4.5-fold among those with tHcy levels of 9–15, 15–20, and $\geq 20\ \mu$M, respectively. A recent study by our group involving the elderly Framingham Study population has demonstrated a twofold increase in all-cause cardiovascular disease mortality among those in the highest quartile of tHcy compared with those in the lowest tHcy quartile (11).

Elevated plasma homocysteine levels were also found to be associated with increased risk of neural tube defects (NTD) (64, 130). A study with a chicken embryo model demonstrated that high homocysteine induces congenital defects of both the neural tube and the heart (117). Other studies demonstrated associations between elevated plasma tHcy and Alzheimer's disease, dementia, and loss of cognitive function (7, 56, 87, 114).

It is important to note that these relations between elevated plasma homocysteine levels and diseases are attained through epidemiological studies. The question of causality remains to be resolved. This chapter reviews homocysteine metabolism with emphasis on its nutritional regulation as it pertains to conditions that are associated with elevated plasma homocysteine levels and discusses current views regarding the mechanism that underlies the relationship between elevated plasma homocysteine levels and vascular disease.

THE HOMOCYSTEINE METABOLIC PATHWAYS

Homocysteine is a non-protein–forming sulfur amino acid whose metabolism is at the intersection of two metabolic pathways: remethylation and transsulfuration (Figure 1). In remethylation, homocysteine acquires a methyl group from N-5-methyltetrahydrofolate or from betaine to form methionine. The reaction with N-5-methyltetrahydrofolate occurs in all tissues and is vitamin B_{12} dependent, whereas the reaction with betaine is confined mainly to the liver and is vitamin B_{12} independent. A considerable proportion of methionine is then activated by ATP to form *S*-adenosylmethionine (SAM). SAM serves primarily as a universal methyl donor to a variety of acceptors. *S*-adenosylhomocysteine

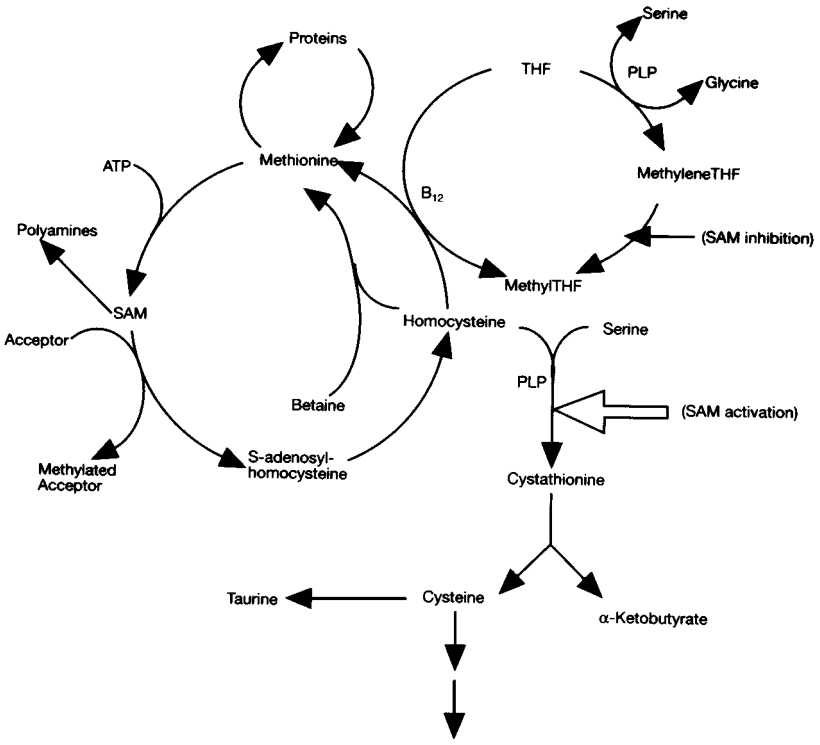

Figure 1 Homocysteine metabolism in man and animals. Large arrows indicate enzyme reactions that are regulated by *S*-adenosylmethionine (SAM); closed arrow indicates inhibition; open arrow indicates activation. Enzymes: N-5-methyltetrahydrofolate, homocysteine methyltransferase; methylenetetrahydrofolate reductase; betaine, homocysteine methyltransferase; choline dehydrogenase; cystathionine β-synthase; γ-cystathionase. THF, tetrahydrofolate; PLP, pyridoxal-5′-phosphate. (From Reference 124.)

(SAH), the by-product of these methylation reactions, is subsequently hydrolyzed, thus regenerating homocysteine, which then becomes available to start a new cycle of methyl-group transfer. It is important to note that this hydrolysis is a reversible reaction that favors the synthesis of SAH, and that elevated cellular concentrations of this metabolite are likely to precede and accompany all forms of hyperhomocysteinemia.

In the transsulfuration pathway, homocysteine condenses with serine to form cystathionine in an irreversible reaction catalyzed by the pyridoxal-5′-phosphate (PLP)–containing enzyme, cystathionine β-synthase. Cystathionine is hydrolyzed by a second PLP-containing enzyme, γ-cystathionase, to form cysteine and α-ketobutyrate. Excess cysteine is oxidized to taurine or inorganic

sulfates or is excreted in the urine. Thus, in addition to the synthesis of cysteine, this transsulfuration pathway effectively catabolizes excess homocysteine, which is not required for methyl transfer.

NUTRITIONAL REGULATION OF HOMOCYSTEINE METABOLISM

Studies of the regulation of homocysteine metabolism have demonstrated that the utilization of homocysteine molecules by the transsulfuration and remethylation pathways is nutritionally regulated. Two studies have shown that when the intake of labile methyl groups (i.e. methionine and choline) is modified, the de novo synthesis of methionine methyl groups is affected (96, 100). When a basal methionine-containing diet was administered, homocysteine moieties were found to cycle through the remethylation pathway approximately 1.5–2.0 times before being catabolized through the transsulfuration pathway. When dietary methionine was halved, the number of cycles per homocysteine moiety increased twofold. Conversely, when excess dietary methionine was administered, homocysteine cycling fell below basal levels. Similar adaptations to changing levels of dietary methionine were observed in rats (36).

This capacity of the body to discriminate between the remethylation and transsulfuration pathways as a way to adapt to varying amounts of methionine in the diet strongly implies the existence of a coordinate regulation between these two pathways. Available experimental evidence, obtained primarily from measurements of enzyme activities in vitro, suggests that this coordination is achieved by at least two mechanisms. The first mechanism is a function of SAM's propensity to act as an allosteric inhibitor of methylenetetrahydrofolate reductase (MTHFR) and as an activator of cystathionine β-synthase (39, 68, 72, 73) (Figures 1 and 2). As such an effector, SAM suppresses the synthesis of an important substrate (N-5-methyltetrahydrofolate) required for remethylation and promotes the initial reaction of transsulfuration (cystathionine synthesis). Thus, intracellular SAM concentration is an important determinant of the fate of homocysteine molecules.

The second mechanism by which remethylation and transsulfuration are coordinated consists of the regulation of intracellular SAM concentration, itself. In the liver, SAM synthesis is catalyzed by two enzymes peculiar to this organ that are immunologically similar but different in other respects (18, 19, 107). One enzyme, a tetramer of high molecular weight, exhibits a high affinity for methionine and is thought to function at normal physiological conditions. The second enzyme is a dimer of a lower molecular weight, has a low affinity for methionine, and is thought to function under conditions of high methionine intake. Thus, changes in intracellular methionine, particularly due to dietary

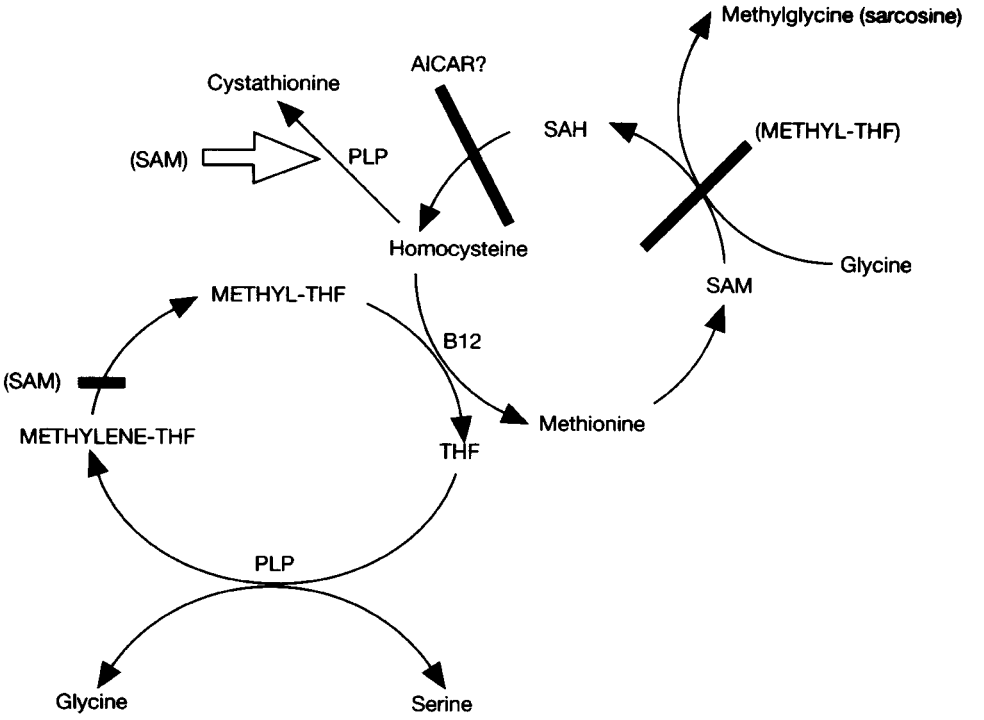

Figure 2 Regulation of methionine methyl group synthesis. (From Reference 124.)

intake, will affect the rate of SAM synthesis based on the activity of the SAM synthetase enzymes.

Additionally, it is also thought that the utilization of SAM is regulated specifically by a reaction in which the methyl group of SAM is transferred to the amino group of glycine, forming sarcosine (Figure 2). This reaction is catalyzed by glycine N-methyltransferase (GNMT), which is abundant in the liver and strongly inhibited by N-5-methyltetrahydrofolate polyglutamates (6, 24, 143). Thus, along with intracellular methionine, N-5-methyltetrahydrofolate participates in the regulation of intracellular SAM concentrations.

When the two mechanisms of regulation are considered together, the following scenarios can be predicted:

1. When dietary methionine is high, the low-molecular-weight SAM synthetase will rapidly convert the incoming methionine to SAM. The resulting rise in intracellular SAM concentration will be associated with (*a*) inhibition of methylenetetrahydrofolate reductase resulting in suppressed N-5-

methyltetrahydrofolate synthesis, thereby allowing the GNMT enzyme to act near full capacity because of suppressed inhibitor (N-5-methyltetrahydrofolate) concentration; and (*b*) activation of the cystathionine β-synthase enzyme, thus increasing the rate of homocysteine catabolism. In this way, homocysteine transsulfuration is promoted over remethylation, consistent with the reduced need for de novo methionine synthesis due to the high dietary supply of methionine.

2. Conversely, when the dietary methionine supply is low, SAM concentration is insufficient for the inhibition of MTHFR, resulting in an elevated rate of N-5-methyltetrahydrofolate production. The resulting rise in intracellular N-5-methyltetrahydrofolate concentration will be associated with (*a*) inhibition of GNMT and thereby conservation of SAM, and (*b*) an increase in the availability of substrate for homocysteine remethylation. Thus, remethylation will be favored over transsulfuration because the concentration of SAM is too low to activate the cystathionine β-synthase enzyme. This process is consistent with the increased need for de novo methionine synthesis attributed to the low dietary input of methionine.

THE PATHOGENESIS OF HYPERHOMOCYSTEINEMIA

The small amount of homocysteine normally found in the plasma is the result of a cellular export mechanism that complements the catabolism of homocysteine through transsulfuration by helping maintain low intracellular concentrations of this potentially cytotoxic sulfur amino acid (22, 137). Barring kidney malfunction, the occurrence of hyperhomocysteinemia indicates that homocysteine metabolism has in some way been disrupted and that the export mechanism is disposing into the blood excess homocysteine that has accumulated in the cell. This prevents toxicity to the cell but leaves vascular tissue exposed to the possibly deleterious effects of excess homocysteine.

Either a genetic defect in one of the enzymes of homocysteine metabolism or a nutritional deficiency of one or more of the vitamins that participate in homocysteine metabolism can lead to metabolic disruption and potentially to hyperhomocysteinemia. The severity and type of the resulting hyperhomocysteinemia is dependent on the extent to which the particular disturbance affects the coordination of the two pathways of homocysteine metabolism. A discussion of these disturbances follows.

Defective Synthesis of N-5-Methyltetrahydrofolate

Synthesis of N-5-methyltetrahydrofolate is the first step specifically concerned with the synthesis of methionine. An immediate consequence of impaired synthesis of this folate, either because of folate deficiency or because of a

defect in MTHFR, is a depressed synthesis of methionine. This leads to the diversion of homocysteine, which was destined for remethylation, toward the transsulfuration pathway. This latter pathway, however, is incapable of handling the additional homocysteine for two reasons. First, the depressed synthesis of methionine will lead to a decrease in intracellular SAM concentration. Second, the lack of N-5-methyltetrahydrofolate will allow GNMT to be fully active, further decreasing the SAM concentration and increasing the synthesis of homocysteine as a by-product of glycine methylation. Thus, the transsulfuration pathway becomes ineffective because of the increased homocysteine burden in conjunction with a concentration of SAM too low to activate cystathionine synthesis, the initial reaction of transsulfuration. As a result, homocysteine accumulates in the cell and subsequently is exported into the blood, causing hyperhomocysteinemia.

Defective Homocysteine Remethylation

In cases of impaired homocysteine remethylation, as in vitamin B_{12} deficiency or defects in any of the methyl-cobalamin synthesis enzymes, conditions and consequences are somewhat different from those of impaired N-5-methyltetrahydrofolate synthesis. The methyl trap hypothesis (52) predicts that N-5-methyltetrahydrofolate will actually accumulate when remethylation is impaired. Therefore, despite the decrease in SAM synthesis due to the B_{12} deficiency or enzyme defect, intracellular SAM concentrations may be less affected because the accumulated N-5-methyltetrahydrofolate will inhibit the utilization of SAM in glycine methylation. As a consequence, less homocysteine will be synthesized from SAM and there will be at least some activation of cystathionine β-synthase. Thus, homocysteinemia that results from impaired homocysteine remethylation may not be as severe as that observed in impaired N-5-methyltetrahydrofolate synthesis because transsulfuration will be somewhat more active in the catabolism of homocysteine. However, it is important to say that hyperhomocysteinemia will occur because homocysteine metabolism is disrupted to a significant extent.

Defective Homocysteine Transsulfuration

No product of the transsulfuration pathway, e.g. cystathionine, cysteine, or taurine, is known to directly affect the remethylation pathway. Nevertheless, the remethylation pathway can be affected by the transsulfuration pathway. When the latter pathway is severely impaired, as in homozygous cystathionine β-synthase defect, there is a diversion of homocysteine toward the remethylation pathway. Under these conditions, the rate of methionine synthesis is increased, leading to a temporal increase in intracellular SAM concentration. This increase

in SAM concentration will continue until the level of this metabolite is sufficient for a feedback inhibition of MTHFR, at which point the remethylation system is inhibited. Consequently, both pathways of homocysteine metabolism are impaired and severe hyperhomocysteinemia results.

When transsulfuration is only mildly impaired, as in vitamin B_6 deficiency or in a heterozygous defect of cystathionine β-synthase, the fully active remethylation pathway and the residual activity of the transsulfuration pathway are sufficient to prevent the precipitation of hyperhomocysteinemia provided the homocysteine burden is low. The homocysteine burden is low when the influx of methionine, the metabolic precursor of homocysteine, is diminished. This occurs under fasting conditions during which there is no dietary input of methionine. However, despite the lack of hyperhomocysteinemia under fasting conditions, there is a disruption of homocysteine metabolism that is detected when the homocysteine burden is increased, i.e. under nonfasting conditions during which there is a significant intake of dietary methionine. This influx of dietary methionine will lead to an increase in intracellular SAM concentration with the following consequences: (*a*) inhibition of N-5-methyltetrahydrofolate synthesis, hence depressed use of homocysteine through remethylation; and (*b*) highly active GNMT because of low intracellular N-5-methyltetrahydrofolate concentration, hence accelerated generation of homocysteine through glycine methylation. Coupled with the primary impairment of transsulfuration due to the B_6 deficiency or to the heterozygous cystathionine β-synthase defect, these conditions lead to the significant impairment of the ability of the homocysteine pathways to metabolize homocysteine, and hyperhomocysteinemia results.

Incidentally, this abnormal rise in blood homocysteine concentration after ingestion of a diet containing methionine is the basis for the oral methionine load test. The methionine load test is a comparison of plasma homocysteine concentrations before and after ingestion of a large dose of methionine, usually 100 mg/kg of body weight. The resulting increase in homocysteine synthesis due to the large influx of methionine will test the capacity of the homocysteine pathways. An abnormal test result is attained whenever the capacity of the pathways to metabolize homocysteine is significantly exceeded by the rate of homocysteine generation. In normal individuals, a small rise in blood homocysteine concentration after the load will be observed that returns to baseline within a few hours. In individuals with vitamin B_6 deficiency or a heterozygous cystathionine β-synthase defect, an abnormal response will be observed consisting of a much larger and more persistent rise in blood homocysteine concentration [defined as at least 2 standard deviations (SD) above the rise observed in a normal individual].

SUPPORTING EVIDENCE FOR NUTRITIONAL CAUSES OF HYPERHOMOCYSTEINEMIA

Using rats as models, we found that fasting plasma homocysteine concentrations increased 8- to 10-fold in folate-deficient rats and 2.5-fold in vitamin B_{12}-deficient rats (Figure 3). In the folate-deficient rats, an inverse correlation was observed between intracellular SAM concentration and plasma homocysteine concentration (Figure 4) (91). Moreover, when the folate-deficient rats were administered intraperitonial (i.p.) injections of ethionine, a methionine analog, plasma homocysteine concentration decreased almost to its normal level (Figure 5). This decrease is thought to be due to *S*-adenosylethionine, which, like SAM, is an effective activator of cystathionine β-synthase, but unlike SAM is less likely to be deethylated and subsequently metabolized to homocysteine (39, 68). This activation by *S*-adenosylethionine reintroduced the coordination

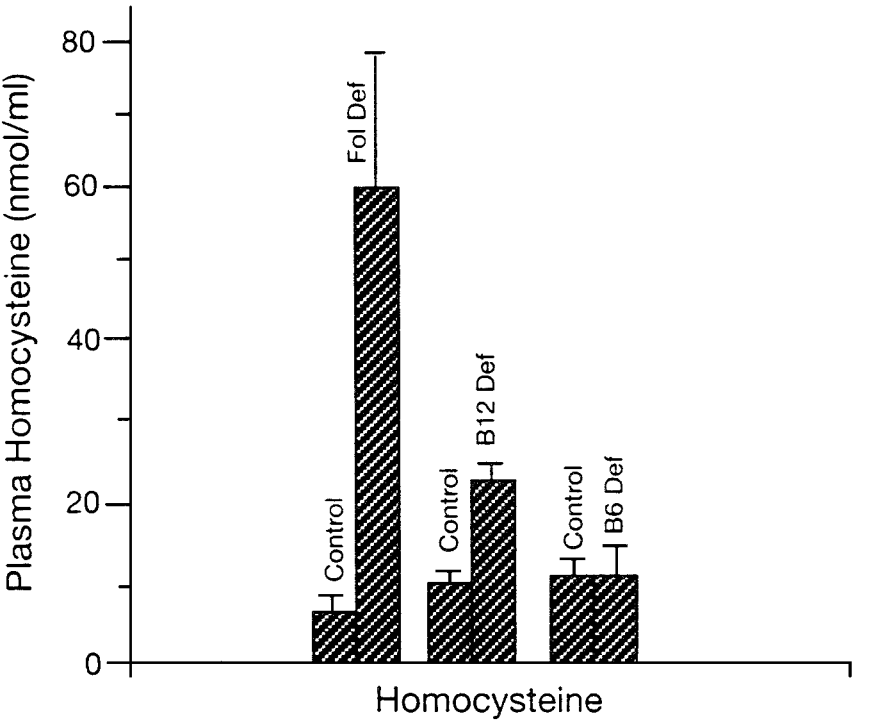

Figure 3 Effects of folate, vitamin B_{12}-, and vitamin B_6-deficiencies on fasting plasma homocysteine levels. (From Reference 124.)

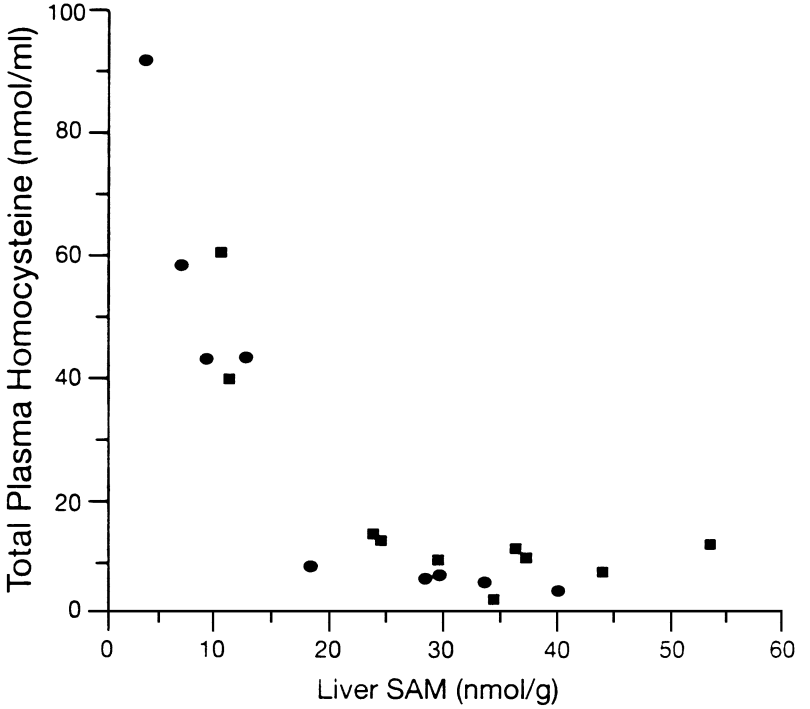

Figure 4 Relationship between fasting plasma homocysteine and hepatic *S*-adenosylmethionino (SAM) concentrations. (From Reference 124.)

between the two pathways that was interrupted in folate deficiency because of diminished SAM synthesis.

In vitamin B_6-deficient rats, fasting plasma homocysteine concentration was not elevated (Figure 3) (92, 127). Moreover, an oral gavage of methionine caused a marked increase in plasma homocysteine concentration in the vitamin B_6-deficient rats that was accompanied by a significant elevation in hepatic SAM concentration (90). This is contrasted with folate-deficient rats in which methionine loading caused no significant change in plasma homocysteine concentration from preload levels (Figure 6) (90).

HYPERHOMOCYSTEINEMIA IN HUMANS

Hyperhomocysteinemia in humans can be distinguished by category, including cause, prevalence, and severity. The more severe cases are due to homozygous

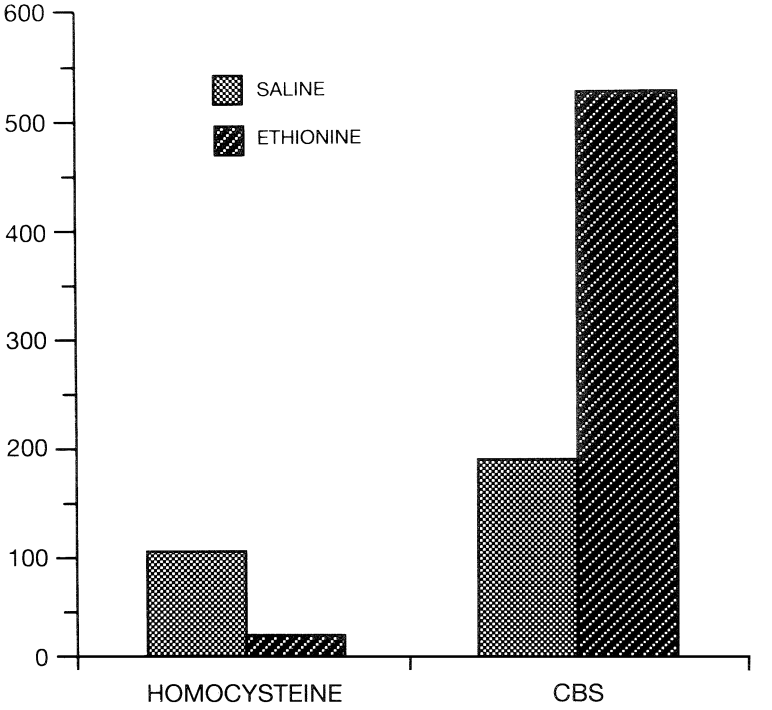

Figure 5 Effect of ethionine administration on fasting plasma homocysteine concentration and hepatic cystathionine β-synthase (CβS) activity in folate-deficient rats. (From Reference 124.)

defects in genes encoding for enzymes of homocysteine metabolism (Table 1). In such cases, a defect in an enzyme involved in either homocysteine remethylation or transsulfuration leads to large elevations of homocysteine in the blood and urine. The classic form of such a disorder—congenital homocystinuria—is caused by homozygous defects in the gene encoding for cystathionine beta synthase (CBS). In these individuals, fasting plasma homocysteine concentrations can be as high as 400 μmol/liter (99). Homozygous defects of other genes that lead to similarly severe elevations in plasma homocysteine include those encoding for MTHFR or for any of the enzymes that participate in the synthesis of methylated vitamin B_{12} (61, 62, 76, 98, 101, 125).

The more common causes of hyperhomocysteinemia are also moderate in character and may be due to less severe defects in genes encoding for enzymes or from inadequate status of those vitamins that are involved in homocysteine metabolism (Table 1). Plasma homocysteine concentrations in these instances may differ, depending on which arm of the two metabolic pathways

Table 1 Classification of hyperhomocysteinemia[a]

Severe hyperhomocysteinemia	High tHcy levels at all times; caused by deficiencies in CBS, MTHFR, or in enzymes of B_{12} metabolism
Mild hyperhomocysteinemia	Fasting; moderately high tHcy levels under fasting conditions; reflects impaired homocysteine methylation (folate, B_{12} or moderate enzyme defects, e.g. thermolabile MTHFR)
Post-methionine load	Abnormal increase in tHcy after methionine load. Abnormal net increase reflects impaired homocysteine transsulfuration (heterozygous CBS defects, B_6 deficiency)

[a] Cystathionine beta synthase (CBS), methylenetetrahydrofolate reductase (MTHFR), total homocysteine (tHcy).

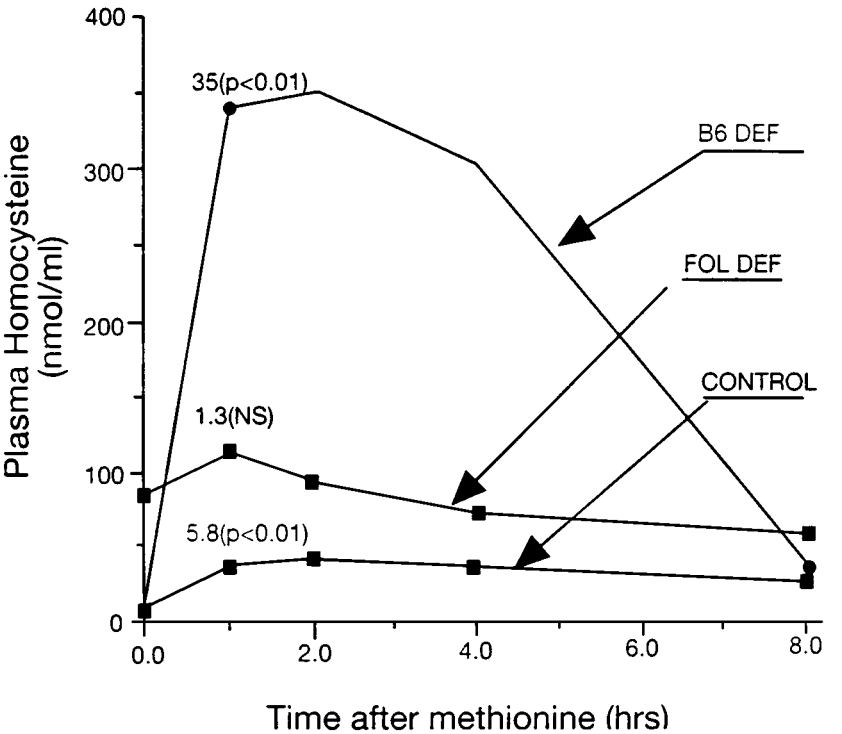

Figure 6 Plasma homocysteine concentrations after methionine loading in control, folate-deficient, and vitamin B_6-deficient rats. (*Parentheses*) fold increase in homocysteine concentrations from baseline. (From Reference 124.)

of homocysteine metabolism is defective (123). An impairment in the remethylation pathway, even if it is mild, will lead to a substantial increase in plasma homocysteine concentrations under fasting conditions. Such an impairment may be due to inadequate status of either folate or vitamin B_{12} or to defects in the gene encoding for MTHFR (4, 14, 17, 31, 33, 54, 58, 60, 75, 77, 78, 123, 128, 147, 148). MTHFR contains FAD as a prosthetic group, which raises the possibility that vitamin B_{12} status is also a determinant of fasting plasma homocysteine levels (126).

In contrast, a mild impairment in the transsulfuration pathway will lead, at most, to a very slight increase in fasting plasma homocysteine levels. This mild impairment, which may be due to heterozygous defects in the CBS gene or inadequate levels of vitamin B_6 (8, 13, 119, 132), is normally identified by an abnormal increase in plasma homocysteine after a methionine loading test or following a meal (90–92, 111, 127).

Evidence of two distinct forms of hyperhomocysteinemia in humans has been derived from preliminary data obtained from 274 consecutive participants in the Family Heart Study (10). Plasma tHcy was measured at fasting and 4 h after a methionine load for each participant. Using homocysteine values greater than 90% for the definition of hyperhomocysteinemia (both fasting and methionine load), it was shown that of 44 hyperhomocysteinemic individuals, 20 (46%) had fasting hyperhomocysteinemia only, 17 (34.5%) had postmethionine load hyperhomocysteinemia only, and just 7 (14%) had both types of hyperhomocysteinemia. Recently, we conducted a placebo-controlled, homocysteine-lowering trial in healthy kidney transplant recipients (9) to show that whereas fasting homocysteine can be lowered by a combination of folate and vitamin B_{12}, post-methionine-load homocysteine can only be lowered by B_6 supplementation.

Existence of an interrelationship between vitamin status and plasma homocysteine was first reported by Kang et al (61), who showed an inverse relationship between homocysteine and plasma folate concentrations. Other studies have shown existence of inverse correlations between homocysteine and folate or vitamin B_{12} plasma concentrations and the efficacy of vitamin supplementation in the lowering of plasma homocysteine levels (26, 41, 46, 47, 49, 54, 56, 58, 59, 65–72, 104, 110, 135, 140). In these latter studies, folate and vitamin B_{12}, but not vitamin B_6, supplementation reduced fasting plasma homocysteine levels. Vitamin B_6 was found to be effective in lowering post–methionine-load plasma homocysteine.

The independent associations between individual nutrients and homocysteine concentrations were studied in an established cohort of Americans, the Framingham Heart Study (Figure 7) (122). After controlling for age, sex, and other vitamins, nonfasting plasma homocysteine exhibited a strong, nonlinear inverse association with plasma folate. Minimum levels of homocysteine were

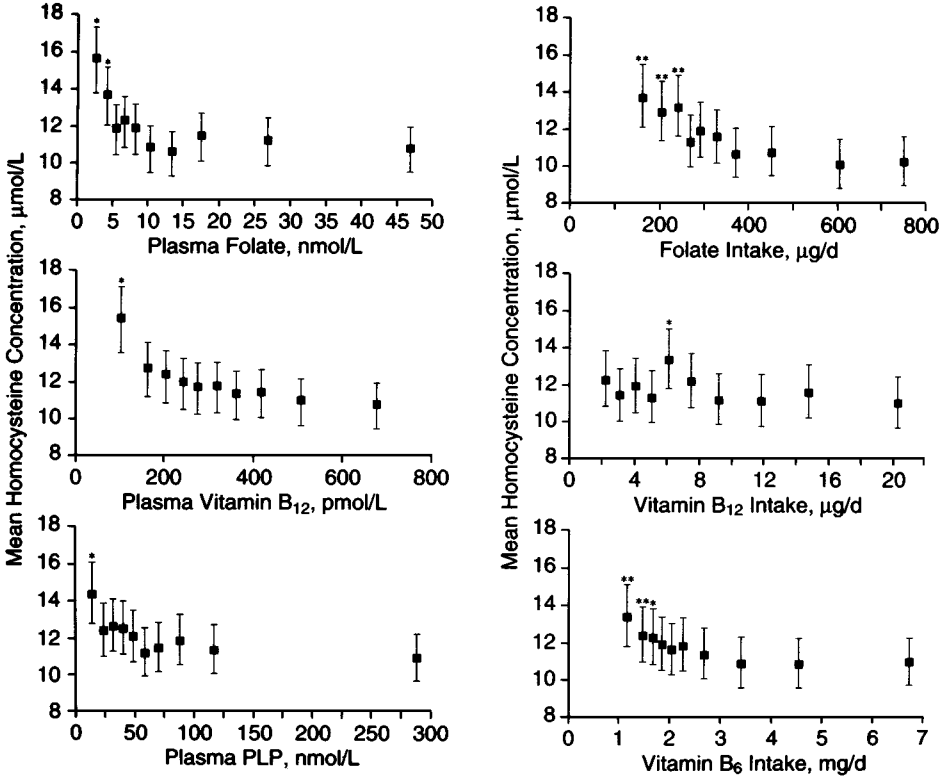

Figure 7 (*Left*) Mean plasma homocysteine concentrations (and 95% confidence intervals) by deciles of plasma folate (*top*), vitamin B_{12} (*center*), and pyridoxal-5′-phosphate (PLP) (*bottom*) concentrations. Means are adjusted for age, sex, and other plasma vitamins. (*Asterisk*) Significant difference from mean in the highest decile ($P < 0.01$). (*Right*) Mean plasma homocysteine concentrations (and 95% confidence intervals) by deciles of intake of folate (*top*), vitamin B_6 (*center*), and vitamin B_{12} (*bottom*). Means are adjusted for age, sex, and other vitamin intakes. (*Asterisk*) indicates significant difference from mean in the highest decile ($P < 0.05$); (*double asterisk*) significant difference from mean in the highest decile ($P < 0.01$). (From Reference 122.)

observed around 10 nmol/liter of folate and above. Nonfasting plasma homocysteine exhibited weaker inverse associations with plasma concentrations of vitamin B_{12} and pyridoxal-5′-phosphate. Plasma homocysteine levels also exhibited inverse association with folate intake and, to some extent, vitamin B_6 intake, but not with vitamin B_{12} intake (Figure 7).

In this elderly Framingham study population (ages 67–96 years), about 30% had elevated plasma homocysteine levels (over 14 μM). Two thirds of the incidences of homocysteine elevations are attributed to inadequate vitamin status or intake.

MTHFR THERMOLABILITY

Relation to Plasma Homocysteine and Folate Status

Mutations that result in severely reduced MTHFR activity and hyperhomocysteinemia are rare (48, 49, 116). However, in 1988, Kang et al (61) reported that two unrelated patients with moderate hyperhomocysteinemia and low folate levels had a variant of MTHFR that was distinguished from the normal enzyme (as measured in lymphocyte extracts) by its lower specific activity (50%) and its thermolability. In subsequent studies, the same group demonstrated that MTHFR thermolability is an inherited recessive trait, which is present in approximately 5% of the general population and 17% of patients with proven coronary artery disease, but it is not associated with neurological complications (57, 59). The cDNA for human MTHFR has recently been isolated (49), and it has been shown that MTHFR thermolability is caused by a point mutation (677C to T transition) at a polymorphic site, resulting in a valine substitution for an alanine in this enzyme (43). The mutation was found in 38% of unselected chromosomes from 57 French Canadian individuals; the homozygous state for the mutation was present in 12% of these subjects and correlated with significantly raised tHcy (43). Preliminary evidence indicates that the frequency of homozygotes for the 677CT mutation may vary significantly in populations from different geographic areas (from 1.4% to 15%) (95).

The impact of MTHFR thermolabile variant on plasma homocysteine levels is unclear. The hyperhomocysteinemia seen in the original patients of Kang et al (61) was associated with low folate plasma levels, and folate supplementation reduced homocysteine to normal levels. In other studies, a large proportion of the population with a TT mutation in the MTHFR gene had normal plasma tHcy levels. Furthermore, the hyperhomocysteinemia seen in those with elevated tHcy was mild and certainly did not correspond with the 50% decrease in enzyme activity seen in vitro with lymphocyte or fibroblast extracts.

This lack of concordance between enzyme activity of the thermolabile MTHFR–determined in cell extracts, and circulating tHcy implied that other factors control the enzyme activity in vivo. In a recent study we demonstrated the occurrence of an interaction between MTHFR thermolability genotype and folate status (55). When plasma folate concentrations were above the median (15.4 nmol/liter), plasma homocysteine levels were low and unrelated to the MTHFR genotype. However, when plasma folate concentrations were below the median, plasma homocysteine levels were significantly higher in homozygotes for the 677CT mutation than in those with the normal genotype (55). The existence of such an interaction has since been confirmed in other studies (2, 21, 121). These data imply that the phenotypic expression of the MTHFR genotypes is dependent on the availability of folate. This suggests

that homozygotes for the thermolabile genotype might have a higher folate requirement than do individuals with a normal genotype.

OTHER FACTORS THAT CONTROL PLASMA HOMOCYSTEINE LEVELS

Plasma homocysteine levels increase with age and are higher in men than in women. High tHcy levels are associated with impaired renal function, high plasma creatinine, smoking, coffee consumption, alcoholism, and certain drugs, including folate antagonists, nitrous oxide, and L-DOPA (112).

PATHOPHYSIOLOGY OF HYPERHOMOCYSTEINEMIA

In spite of the large number of studies indicating that mild elevations of homocysteine in plasma are associated with an increased risk for occlusive vascular disease, thrombosis, and stroke, the question of whether homocysteine per se is responsible for these associations remains unanswered. A survey by Mudd et al (97) of the parents and grandparents of homocystinuric children concluded that heterozygosity for CBS deficiency is not associated with increased risk in heart attacks and stroke (97). Swift & Morrell (131) questioned the validity of some of the methods used by Mudd et al and argued that the data actually point to increased mortality rates in this heterozygote population. Two studies that employed a noninvasive (doppler ultrasound) technique also provided conflicting results. In one, no evidence was found of increased frequency of endothelial plaques in the neck arteries of 25 Irish heterozygotes, compared with 21 control subjects (23). Another study, however, indicated more-frequent early vascular lesions in the iliac and internal carotid arteries in 14 heterozygotes than occurred in 47 controls (118). A study at the molecular biology level (70) examined the CBS alleles in four patients with premature occlusive arterial disease who were (*a*) hyperhomocysteinemic based on post-methionine-load results and (*b*) had lower enzyme activity in their fibroblasts (70). None of the eight alleles contained any mutation that resulted in diminished enzyme activity. In a prior study, this same group demonstrated that cultured fibroblasts are not always reliable for testing the phenotypic expression in homocystinuric patients (71). In other studies it was found that prevalence of the more common CBS mutations is not higher in the patient populations (65). In another report, a knockout mouse with CBS deficiency was found to lack manifestations of thrombosis or/and cardiovascular complications and instead exhibited fatty livers (145). This is in spite of the fact that the levels of homocysteine in plasma were 40-fold higher in the homozygote mice than in the control mice. Other inconsistencies relate to the question of whether MTHFR thermolability confers increased risk for

the various diseases. In an earlier study that relied on enzyme activity, a higher prevalence (17%) of this variant was found in a North American CAD population than in healthy control subjects (5%) (57). Similarly, in a Dutch population the prevalence of the 677CT homozygotes was higher in vascular disease patients (15%) than in control subjects (5%); and in an Italian patient population, the prevalence was 29.7% compared with only 15.1% in healthy control subjects (29, 65). A homozygous frequency of 17% in 111 patients with coronary artery disease compared with only 7% in 105 control subjects was reported in Ireland (45). A study conducted in Brazil demonstrated higher prevalence of the 677CT homozygotes in 191 patients with arterial disease (19%) and in 127 patients with venous thrombosis (11%) compared with 296 unmatched control subjects (4%) (5). In Japan, a higher prevalence of the homozygous 677CT mutation was found in 362 patients with coronary artery disease (15.7%) than in 778 controls (10.2%, $P = 0.001$) (94). Furthermore, in patients with triple vessel disease, the prevalence of the 667CT homozygous mutation was 26% compared with only 14% and 15% prevalence in patients with a single or a double vessel disease. Other studies, however, have been contradictory. No difference in the prevalence of the 667CT homozygous mutation was found between coronary artery disease patients and control subjects (see ref 146 for a mini review) in populations from the United States and Australia (120, 149), in a second Dutch population (101), in a French Canadian population (2), in a British population (141), in the Physician Health Study (82), and most recently among women with cardiovascular disease in the United States (121). Of the 17 additional studies that were published in 1998, only four suggested that MTHFR thermolability is a risk factor of vascular disease. The remaining studies found no such association (1, 3, 20, 30, 34, 38, 40, 47, 66, 69, 83, 84, 93, 103, 108, 113, 142).

A recent nested–case-control study of men participating in the Multiple Risk Factor Intervention Trial (MRFIT) found no significant differences in plasma homocysteine levels between control subjects and case patients who had nonfatal myocardial infarctions that occurred within 7 years of sample collection, nor in CHD deaths that occurred more than 11 years after sample collection (40).

Some of these inconsistencies can be explained by differences in genetic background and dietary habits of the study population, or by differences in the pathology among species (e.g. human beings vs mice). Improper selection of the population representing control subjects is likely to account for some of the inconsistencies in the prevalence of the 677CT mutation between patients and control subjects. This may be the case in the original study that reported only a 5% prevalence of the thermolabile MTHFR in the control population (43), and in the first Dutch study, which also reported a 5% prevalence in the control group (29, 65). In other studies from those same countries, prevalence of the

677CT mutation within the control population was reported to be significantly higher. The fact that in the Brazilian study the cases and control population were unmatched (5) casts doubt on the significance of the findings. However, other data that show clear differences in prevalence of the 667CT homozygous mutation between patients and controls (45, 94) cannot be discounted. Rather, these data point to the possibility that the 677CT mutation is indeed a risk factor for vascular disease in certain regions of the world (e.g. Japan, Ireland, and perhaps Brazil). But for the proper interpretation of other inconsistencies, an understanding of the mechanism that underlies the relationship between homocysteine and disease is still required.

It is generally held that different mechanisms are responsible for arterial and venous thromboembolic diseases, and that these mechanisms involve platelet function abnormalities in arterial thrombosis and abnormalities of coagulation and/or fibrinolysis in venous thromboembolism. Ex vivo studies looking for such abnormalities in patients with hyperhomocysteinemia have provided inconclusive results (46, 51, 53, 85, 89, 102, 109, 138). In subjects with severe hyperhomocysteinemia due to homozygous CBS deficiency, an abnormally high in vivo biosynthesis of thromboxane A2—as reflected by the urinary excretion of its major metabolite 11-dehydro-thromboxane B2—has been observed (32).

Administration of aspirin inhibits thromboxane production; urinary appearance returns to baseline levels over a time course consistent with platelet survival, which suggests that platelets are the major source of increased thromboxane urinary excretion. Because thrombin is a potent inducer of platelet activation, the presence of hypercoagulable state was investigated in homocystinuric patients (25). Increased levels of prothrombin fragment 1.2, thrombin-antithrombin complex, and activated protein C were all observed in homocystinuric patients being treated with vitamins who were free of vascular disease. These abnormalities, however, did not correlate with urinary thromboxane excretion. It is interesting to note that protein C levels, but not factor VII and factor II levels, were significantly reduced in homocystinuric patients and correlated with the degree of hyperhomocysteinemia (25). Diet-responsive deficiency of factor VII was previously reported in CBS-deficient patients (15, 85, 109). Reduced protein C levels may at least partly contribute to venous thrombotic manifestations of patients with homozygous CBS deficiency. The observation that the increased urinary thromboxane excretion was independent of homocysteine levels and was present both in vitamin B_6-responsive and -nonresponsive patients may have an impact on treatment of hyperhomocysteinemia (25). It is noteworthy that although the effectiveness of vitamin B_6 in preventing thromboembolism in pyridoxine-responsive patients was shown statistically to be highly significant, the occurrence of thromboembolism was not abolished by vitamin supplementation (37).

Many other studies on the mechanism of hyperhomocysteinemia-related disease employed cell culture systems to show a variety of deleterious effects caused by incubating these cells with homocysteine (28, 86). These effects include the following: inhibition of prostacyclin synthesis, activation of factor V, inhibition of protein C activation, down-regulation of thrombomodulin expression, and blocking of binding of tissue plasminogen activator (t-PA) (but not plasminogen) to endothelial cells. The toxic effect of homocysteine also results in increased platelet adhesion, impaired regulation of endothelium-derived relaxing factor and related nitrogen oxides, induction of tissue factor, suppression of heparan sulfate expression, stimulation of smooth muscle cell proliferation, and oxidation of low-density lipoprotein.

A major shortcoming of these observations is the uncertainty with regard to their pathophysiological significance. Human plasma contains two sulfur-containing amino acids, homocysteine and cysteine, at respective mean normal concentrations of 10 and 240 μmol/liter. In mild hyperhomocysteinemia, the levels of homocysteine are on average 30% higher than normal; as opposed to those rare, severe cases of homocystinuria, where the levels may be as high as 200-400 μmol/L (76, 99). Because cysteine appears to be harmless as far as relationship to disease is concerned, these facts strongly imply that the putative action of homocysteine on the blood vessel wall and the coagulation system is stereospecific, involving the entire molecule and taking place at low concentrations (in the micromolar range).

These considerations were not addressed in the various studies on mechanisms. The homocysteine concentrations (1–10 mmol/liter) employed in these studies often exceeded the levels encountered even under the most severe pathological conditions. The possibility that the observed effects seen in these studies were due to nonspecific reactivity of the sulfhydro group of the homocysteine molecule could not be ruled out. Indeed, in several studies where other thiol-containing compounds such as cysteine and mercaptoethanol were tested, the effects were found to be similar to these seen with homocysteine (for an example see 44; see also 67, 105, 129, 134).

More encouraging results derive from recent studies investigating the impact of moderate hyperhomocysteinemia on a number of hematological parameters in both human beings and laboratory animals. In one important study, high-resolution vascular ultrasonography was used to study endothelium-dependent vasolidilation (induced by hyperemia) and endothelium-independent vasolidilation (induced by the injection of nitroglycerin) in nonatherosclerotic peripheral conduit arteries of 26 elderly subjects with hyperhomocysteinemia (tHcy $=$ 19.2) and 15 age- and sex-matched normohomocysteinemic control subjects (tHcy $=$ 8.2 μM) (133). The data demonstrated that the endothelium-dependent, but not the endothelium-independent, vasolidilation was significantly

lower in the hyperhomocysteinemic group than in the control group. These data were corroborated in a second study that used a cymonologus monkey model to investigate possible mechanisms of action of mild hyperhomocysteinemia (74). Mild hyperhomocysteinemia was induced by a diet high in methionine, depleted of folate, and free of choline. Total homocysteine concentrations were 10.6 μM in the experimental monkeys and 4.0 μM in the control group. In response to activation of platelets by infusion of collagen, blood flow to the leg decreased by 42% compared with 14% in the control group. The response of resistance vessels to the endothelium-dependent vasodilators, ADP and acetylcholine, was markedly impaired in the hyperhomocysteinemic monkeys, which indicates that increased vasoconstriction in response to collagen may be caused by decreased vasodilator responsiveness to platelet-generated ADP. Furthermore, thrombomodulin anticoagulant activity in aorta decreased by 34% in the hyperhomocysteinemic monkeys.

The endothelium-dependent vasolidilation is a function of the relaxing action of nitric oxide on the blood vessel wall, and demonstration of impaired vasolidilation is important as evidence that homocysteine interferes with this action. This possibility was supported by recent studies (63, 80, 139) that used bovine aortic endothelial cells to show that homocysteine treatment is associated with a dose-dependent decrease in NO levels (63, 80, 139).

This decrease in level was not due to decreased activity of endothelial nitric oxide synthase (eNOS) or to decreased activity in nos3 transcription, which suggests that the decrease in NO levels was not due to decreased synthesis. On the other hand, homocysteine treatment was found to cause a decrease in glutathione peroxidase activity. The authors (63, 80, 139) proposed that homocysteine is a prooxidant that produces H_2O_2 and that under conditions of depressed glutathione peroxidase activity, a greater susceptibility of NO to oxidative inactivation is possible.

Some of these effects of homocysteine on NO and glutathione peroxidase levels in endothelial cell cultures (63) were seen at 10 μM DL-homocysteine minimal concentrations and L-cysteine appears not to be effective in these respects. Nevertheless, the idea that homocysteine is functioning as a prooxidant is not in line with its sulfhydro moiety, which is, rather, considered to be a strong reducing group. A study of the oxidative modification of low-density lipoprotein catalyzed by copper ions, azo compounds, or mediated by mononuclear cells found homocysteine to be ineffective as a prooxidant and suggested that the putative homocysteine-induced atherosclerosis may be explained by mechanisms other than oxidative modification (50).

A study with rats on the effect of folate deficiency on platelet and macrophage activities was done (35). The deficient animals, who were also hyperhomocysteinemic, had peritoneal macrophages with a 20-fold greater tissue factor

activity than the control animals. Folate depletion was also associated with enhanced ADP- and thrombin-induced platelet aggregation. Moreover, the deficient animals exhibited enhanced thromboxane biosynthesis, and there was evidence of diminished unsaturated index in plasma due to a marked fall in long-chain (n-3) polyunsaturated fatty acids. The authors suggested that folic acid deficiency is associated with an increase in oxidative stress, as indicated by an increase in plasma lipid peroxidation and by enhanced susceptibility of erythrocytes to free radicals.

In another recent study, rats were fed a diet containing increasing amounts of homocysteine. The study demonstrated that high plasma homocysteine (8 and 15.2 μM compared with 5.2 μM) is associated with increases in cyclin-dependent kinase in the aortic tissue, lower plasma angiotensin-converting enzyme activity, and higher von Willebrand factor (81).

Rolland et al (115), showed that the hyperhomocysteinemia in minipigs induced by a methionine-rich caseinate diet (9.67 vs 5.64 μM total homocysteine) induced vascular alterations that favor the viscous component of the wall rheology in favor of the elastic component. More important, these alterations share the therapeutic effects by angiotensin-converting enzyme inhibitors in association with hydrochlorothiazides against the atherogenic activation of elastinophylic processes.

Although additional studies are required, these emerging data strongly imply that homocysteine may interact with a variety of systems, resulting in different outcomes. Interaction with endothelial cells may result in the impairment of the plasminogenic nature on account of increased thrombogenic properties. On the other hand, interaction with components in the vascular smooth muscle cells may result in enhanced proliferation of these cells and will result in an increased atherogenic tendency (27, 144). However, the possibility that homocysteine is, rather, an indicator of an aberrant metabolism such as localized folate metabolic abnormalities, for example, should still be considered.

TREATMENT OF HYPERHOMOCYSTEINEMIA

Elevations in plasma homocysteine are common in the general population, particularly in the elderly. Vitamin status is a primary determinant of mild-to-moderate hyperhomocysteinemia and accounts for approximately two thirds of all such cases (132). Vitamin supplementation results in near normalization of plasma homocysteine in most cases (16, 42, 79, 136). The meta-analysis of 27 studies by Boushey et al (12) was concerned with the evaluation of the risk of hyperhomocysteinemia for arteriosclerotic vascular disease, with estimation of the reduction of homocysteine levels by folic acid administration, and with the potential reduction of coronary artery disease mortality by increasing folic acid

intake. These analyses proposed that elevations of total homocysteine were an independent graded risk factor for arteriosclerotic vascular disease and calculated that folic acid fortification of food would reduce the annual mortality of the US population by 50,000. Vitamin supplementation may also reduce recurrence of venous thromboembolic disease in patients with hyperhomocysteinemia. The clinical efficacy of this approach has not been tested, however. In addition, the bulk of evidence indicates that fasting total homocysteine determinations may identify up to 50% of the total population of hyperhomocysteinemic subjects. Patients with isolated methionine intolerance may benefit from vitamin B_6 supplementation.

The time is ripe for a placebo/controlled multicenter trial for determining the efficacy of vitamin supplementation in the reduction of morbidity and mortality among patients with occlusive vascular disease, stroke, and thrombosis. Because vitamins are relatively inexpensive, there is little incentive on the part of drug companies to support such a trial, and it is up to government agencies to assume this task. For these reasons it is important that the design of such a trial take into account all the information available. Homocysteine metabolism requires the participation of folate as well as vitamin B_{12} and vitamin B_6 coenzymes. Reduction of homocysteine levels in plasma requires that all three of these vitamins be supplemented.

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