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Hyperhomocysteinemia and the Endocrine System: Implications for Atherosclerosis and Thrombosis

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I. Introduction

HOMOCYSTEINE (H(e)) is a nonprotein-forming, thiol-containing amino acid formed by demethylation of methionine. It is metabolized by remethylation to methionine or by transsulfuration to cysteine. An elevated plasma H(e) level may occur as a result of inherited disorders, which alter enzyme activity in the transsulfuration and remethylation pathways. Alternatively, nutritional deficiencies of essential cofactors or enzyme substrates, including cobalamin (vitamin B_{12}), folate, or pyridoxine (vitamin B_6), can result in blockade of H(e) metabolic pathways. An elevated plasma H(e) level has recently been established as an independent risk factor for thrombosis and vascular disease (1–11). However, the relationship between hyperhomocysteinemia [HH(e)] and cardiovascular disease remains controversial. Although some prospective studies have confirmed that H(e) is an independent risk factor for cardiovascular disease (3, 6), other studies have not found such a relationship (12, 13). Because of the possibility of reducing plasma H(e) with vitamin therapy, this issue has received considerable public attention. However, no large clinical trials have demonstrated a reduction in cardiovascular risk. Ongoing clinical trials are examining the possibility that vitamin therapy to lower H(e) levels may prevent cardiovascular disease.

Homocystinuria is an inherited disorder characterized by severely elevated plasma H(e). Homocystinuric children are known to develop premature vascular disease involving all major blood vessels (14, 15). McCully (16) first drew attention to a possible link between elevated plasma H(e) and vascular disease, making the seminal observation that extensive arterial thrombosis and atherosclerosis commonly occurs in children with homocystinuria. Boers *et al.* (17) highlighted the association between accelerated vascular disease and moderate elevation in plasma H(e), without the other manifestations of homocystinuria. Since then, there has been considerable interest in mild HH(e) as a risk factor for coronary artery disease, stroke, and peripheral vascular disease. The understanding of the different etiologies of HH(e) is changing because of the ability to discriminate between the types of mutations present in inherited disorders, the ability to distinguish between homozygous and heterozygous mutations, and recognition of factors that modify H(e) metabolism.

A mutation resulting in a thermolabile variant of the meth-

ylene tetrahydrofolate reductase (MTHFR) enzyme is common, occurring in one-third to one-half of alleles and varying slightly with the population studied. In the homozygous state, the thermolabile variant is found in about 8% of the population (1, 2, 18, 19). Mild fasting hyperhomocysteinemia has only been reported in individuals homozygous for this polymorphism (2) and, as discussed below, occurs only with concomitantly low folate levels. Increased cardiovascular risk is not associated with the mutation *per se*. However, the risk of cardiovascular disease is increased in homozygotes with concomitantly low folate levels. A substantial proportion of patients with cardiovascular disease have post methionine load HH(e), suggesting a possible defect in cystathionine-β-synthase (CBS) action. However, mutations of CBS are relatively rare, occurring in only approximately 9% of the population (2).

Thus, the high frequency (25–30%) of postmethionine load HH(e) that occurs in patients with cardiovascular disease again suggests that most cases of HH(e) are due to nongenetic factors. Two possible such factors are nutritional status and hormonal changes. Motulsky (2) highlighted a potential interaction between nutritional and genetic factors, an example of a gene-environment interaction.

The purpose of this review is to update the practicing endocrinologist on methionine-homocysteine metabolism, H(e) measurements, genetics of HH(e), and mechanisms of vascular disease in HH(e). In particular, we will highlight the evidence of interactions between the endocrine system and H(e) metabolism.

II. Methionine-Homocysteine Metabolism

A. Methionine metabolism

Methionine is converted to H(e) through two intermediates: *S*-adenosyl-methionine (SAM) and *S*-adenosyl-homocysteine. The metabolism of H(e) (Fig. 1) occurs either via the transsulfuration pathway or the remethylation pathway (20). It is likely that the remethylation is active in the fasting state and that transsulfuration is predominant after a methionine load such as a high protein meal (see below). H(e) irreversibly condenses with serine to form cystathionine (reaction 1); this reaction is catalyzed by CBS and is also dependent on pyridoxal-5′-phosphate (the active metabolite of vitamin B_6) as a cofactor. Cystathionine is hydrolyzed to cysteine by the enzyme cystathionase (reaction 2) and is also a B_6-dependent reaction. Alternatively, methionine may be reformed via the remethylation pathway when a methyl group is donated to H(e). In this pathway, 5,10-methylene tetrahydrofolate is converted to *N*-5-methyl tetrahydrofolate (reaction 3a), in a reaction catalyzed by MTHFR, with riboflavin as a cofactor. *N*-5-methyl tetrahydrofolate then donates a methyl group to H(e) in a reaction catalyzed by 5-methyltetrahydrofolate-homocysteine methyltransferase (methionine synthase) and its cosubstrate B-12 (reaction 3b). Alternatively, the methyl group may be donated by betaine (reaction 4) in a reaction catalyzed by betaine-homocysteine methyltransferase, forming dimethylglycine and methionine. The betaine-homocysteine reaction is neither vitamin B_{12} nor folate dependent.

B. Regulation of remethylation and transsulfuration of H(e)

As described above, H(e) is metabolized by at least two pathways. Selhub and Miller (21) proposed that the partitioning of H(e) between *de novo* methionine synthesis and catabolism through the cystathionine synthesis occurs by coordinate regulation by SAM (21). They have proposed the hypothesis that impairment of one H(e) metabolic pathway must be associated with the impairment of the other metabolic pathway to cause HH(e). However, this mechanism has only been demonstrated in mice. Proof of coordinate regulation in higher mammals is needed.

Dietary and metabolically derived methionine is conjugated by ATP to form SAM. SAM serves primarily as a methyl donor to a variety of acceptors, including guanidinoacetate, nucleic acids, neurotransmitters, phospholipids,

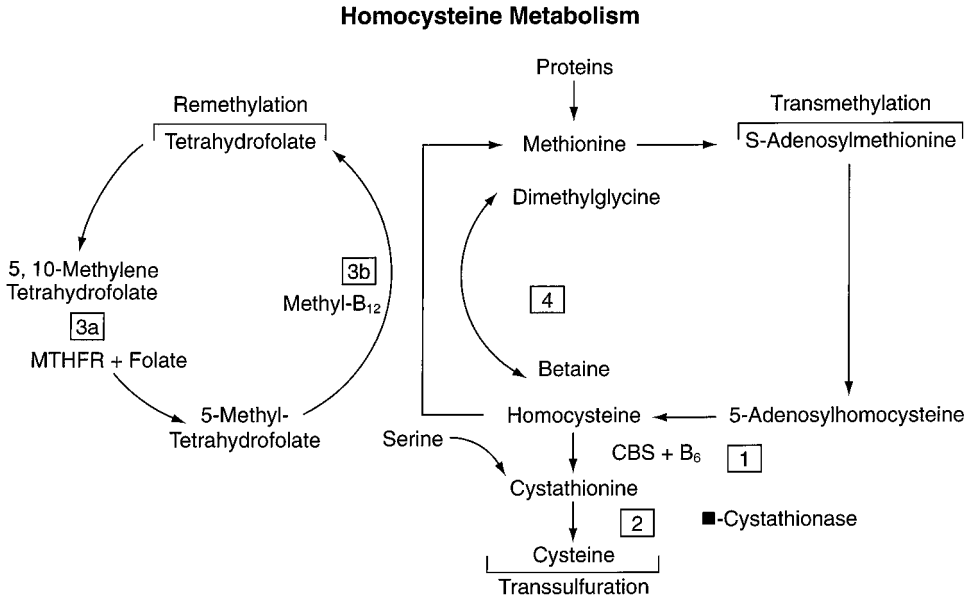

FIG. 1. Summary of the metabolic pathways in homocysteine metabolism. Reaction 1 is catalyzed by choline oxidase; reaction 2, betaine-homocysteine methyltransferase; reaction 3, 5-methyltetrahydrofolate-homocysteine methyltransferase; reaction 4, phosphatidylethanolamine methyltransferase. [Modified from S. Guba *et al.*: *Am J Clin Pathol* 106:709–721, 1996 (25). © 1996 by the American Society of Clinical Pathologists. Reprinted with permission.]

and hormones (21). Creatine synthesis accounts for a major portion of SAM consumption. *S*-adenosylhomocysteine is the byproduct of these methyl transfer reactions and is hydrolyzed to form H(e), which then starts a new cycle of methyl group transfer. In one study, red cell SAM levels were found to be low in patients with coronary artery disease (22). However, red cell SAM may not adequately reflect levels in other tissues, such as the liver, which may be metabolically more active (21).

Studies in rodents have demonstrated that SAM is both an allosteric inhibitor of MTHFR (23) and an activator of CBS (24). Selhub and Miller (21) have proposed that the ability of SAM to act as an enzymatic effector provides a means by which remethylation and transsulfuration can be coordinated. When cellular SAM concentration is low, CBS will be suppressed, resulting in increased remethylation of H(e) for methionine synthesis. Conversely, when SAM concentration is high (as occurs after a methionine load), inhibition of methionine synthase is accompanied by diversion of H(e) through the transsulfuration pathway by stimulation of CBS. Figure 2 illustrates the hypothetical regulation of the metabolic pathways by SAM.

Thus, SAM levels may be the key determinant of plasma H(e). Further investigation is needed on the environmental, hormonal, and other factors that affect SAM and its subsequent effects on H(e) metabolism.

III. Nomenclature and Methodology in the Measurement of Plasma H(e)

We refer the reader to a recent review for details of sample collection (25). Blood should be collected in anticoagulant. EDTA is preferable but heparin or sodium citrate can be used. Plasma H(e) is moderately stable at 22 C and is stable for several weeks at 0–2 C. The plasma can be stored at −20 C; however, repeated thawing should be avoided (26–28). Plasma samples stored for several years have been used in some retrospective studies, and the validity of H(e) measurements in such stored plasma samples remains to be determined.

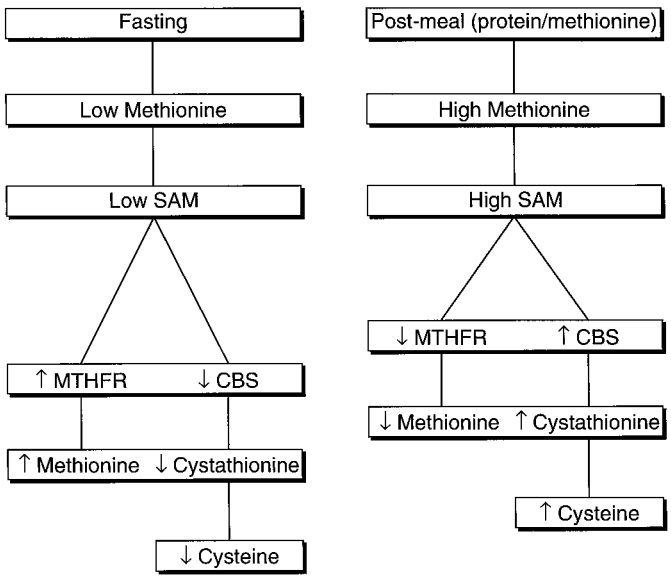

FIG. 2. Regulation of pathways of H(e) metabolism by *S*-adenosylmethionine (SAM). Activation of either pathway reduces H(e) levels.

Homocysteine is the reduced (sulfhydryl) form, and homocystine is the oxidized (disulfide) form of the homologs, cysteine and cystine. For the purpose of this review, both forms of "homocyst(e)ine" will be referred to as the H(e) and hyperhomocyst(e)inemia will be referred to as HH(e). There is confusion between the American and European literature in the abbreviations used: H(e) is abbreviated as Hcy or tHcy in the European literature and H(e) in the American literature. It may be important to achieve consensus in nomenclature and use of abbreviations in this field. In patients in whom H(e) levels are normal, about 70–80% of the total H(e) is bound to protein by a disulfide linkage. With elevated H(e) levels, the percentage of H(e) in the sulfhydryl form represents an increasing percentage of the total H(e) concentration and can increase to 10–25% of the total H(e) (26).

The total H(e) is measured as the free thiol, which is obtained by reduction. This is accomplished by treatment with reducing agents such as sodium borohydride, butylphosphine, or monobromobimane (25–28). Methods used to assay H(e) include gas chromatography with mass spectroscopy, HPLC with or without fluorescence detection, and HPLC with electrochemical detection (25, 26). Methods with HPLC coupled to electron capture detectors do not require derivitization (29). There have been new developments for measuring H(e) that will allow more laboratories to measure these metabolites using immunological analyses, including an enzyme-linked immunoassay and an automated fluorescence polarization analyzer (IMX Abbott Diagnostics, Chicago, IL) (30, 31). Quality controls for the standardization of plasma H(e) measurements are not widely available. Coefficients of variation (CV) for intraassay range between 2 and 8% and for interassay the CVs are between 2 and 10%. Studies on a small population for 4 weeks have shown that within-person variance for a 30-month period showed a high reliability coefficient, but the value was within the accepted range for the most commonly measured chemistry analytes (32).

A. Methionine load test

The methionine load test (MLT) is essential in the comprehensive assessment of HH(e) because heterozygotes for CBS deficiency have abnormal methionine load test results in the setting of normal fasting H(e) levels (16). Conclusive evidence that an abnormal MLT represents an abnormality of the transulfuration pathway is lacking in humans. However, this hypothesis is supported by the data of Dudman *et al.* (33) demonstrating decreased CBS activity in most subjects with an abnormal MLT. Bostom *et al.* (34) have emphasized the importance of the MLT in diagnosing HH(e) in patients with vascular disease. A large proportion of such patients have normal fasting plasma H(e) with an abnormal MLT; and the rate of detection of HH(e) increases significantly when both fasting and postload HH(e) levels are assessed in a study population. The MLT is performed after an overnight fast; blood samples are collected immediately and 2–8 h after a 100 mg/kg methionine load. An abnormal load

test results in a peak plasma H(e) level more than 2 SDs above normal control levels.

IV. Determinants of Plasma Homocysteine

A. Physiological

Several environmental factors have been found to play a role in determining the presence or absence of HH(e) (35, 36). Lussier-Cacan *et al.* (36) studied a large number of healthy men and women, excluding individuals with major and common disorders. They determined that gender was a major determinant of fasting plasma H(e) concentration and that women had a 21% lower concentration than men. The gender difference in H(e) concentrations between men and women persist in elderly persons, although postmenopausal women have higher concentrations than premenopausal women. Plasma H(e) concentrations increase with age and remain an independent risk factor for vascular disease in the elderly (37). The marginal folate and other vitamin deficiencies known to be common in the elderly are likely to be contributing factors to HH(e) (38, 39). There are significant negative correlations between plasma H(e) and serum folate and vitamin B_{12} concentrations. Plasma H(e) was also highest in individuals in the lowest quartile of serum pyridoxal-5′-phosphate, although this active metabolite of vitamin B_6 is more important in determining postmethionine load plasma H(e) than fasting H(e) (36).

Positive correlations have also been found between plasma H(e) and uric acid and creatinine concentrations that may be related to the links between H(e) metabolism with those of creatinine and uric acid (35). Plasma albumin concentration also correlates with plasma H(e) and may reflect an increase in protein-bound H(e). The exact significance of protein binding of H(e) with respect to cardiovascular disease is unknown.

In the Hordaland H(e) study, elevated plasma H(e) was associated with male gender, increasing age, smoking, hypertension, elevated cholesterol, and lack of exercise (40). In a multivariate analysis, Malinow *et al.* (41) demonstrated that systolic blood pressure, plasma uric acid, and hematocrit were predictors of concentrations of plasma H(e) in men who did not have a history of atherosclerotic disease.

It is possible that H(e) in plasma is an "acute phase reactant," rising after vascular injury. Plasma H(e) concentrations rise acutely immediately after a stroke and then decrease over several weeks (42). In contrast, plasma H(e) concentrations tend to be lower immediately after a myocardial infarction (MI) than 6 weeks later (43). The reason for this discrepancy is not clear.

Several other disease states and medications also cause elevations in plasma H(e). The recently described association between HH(e) and diabetes mellitus is described in detail below. These associated factors are outlined in Table 1. The role of genetic mutations, acute events, nutritional status, and hormonal effects are discussed below.

In summary, many nongenetic factors alter plasma H(e) levels either independently or by exacerbating genetic abnormalities in the enzyme.

TABLE 1. Causes of elevated plasma H(e) levels

Nutritional deficiencies
Folate
Vitamin B_{12}
Vitamin B_6
Medications
Methotrexate
Phenytoin and carbamazepine
Nitrous oxide
Theophylline
Metformin
Colestipol and niacin
Disease states
Chronic renal failure
Acute lymphoblastic leukemia
Malignancies
Hypothyroidism
Type 2 diabetes
Type 1 diabetes and nephropathy
Genetic
Transsulfuration abnormalities: cystathionine β-synthase deficiency
Remethylation disorders (*e.g.*, defective vitamin B_{12} transport or coenzyme synthase, defective methionine synthase)
Mutation in MTHFR
Physiological
Age

B. Genetics of hyperhomocysteinemia

Numerous enzyme mutations associated with HH(e) have been described including 17 CBS mutations (reaction 1) and 10 MTHFR point mutations (reaction 3a). One of the mutations for MTHFR, a common polymorphism that is present in one-third to one-half of alleles, results in a thermolabile variant of the MTHFR enzymes (1). The enzyme 5-methyltetrahydrofolate-homocysteine methyltransferase (reaction 3b) (methionine synthase) has been shown to contain one common polymorphism, but no correlation has been found between H(e) levels and genotype. Mutations of the cobalamin coenzyme synthesis enzymes (Cb C, D, E, F, or G) (20) that impair the formation of the cosubstrate methyl-B_{12} (reaction 3b) are also rarely involved in HH(e). A comprehensive review of cobalamin coenzyme synthesis enzyme mutations is available elsewhere (44). The characteristics of CBS and MTHFR mutations are summarized in Table 2. Functional mutations are defined as those associated with increased H(e) levels. Whether any of these mutations *per se* directly increase the risk of arterial or venous occlusive disease remains an area of debate. Most reported studies have not evaluated the genotype of study subjects and have only correlated elevated H(e) levels with risk of vascular occlusive disease. To determine whether genotype is related to the risk of vascular disease, future studies need to correlate genotype, vitamin status (folate, vitamin B_6, pyridoxal phosphate, vitamin B_{12}), H(e) level, and vascular events. These studies may eventually show that genotype does not directly contribute to the risk of vascular disease. Rather, by increasing vitamin requirements, genotype may indirectly affect the risk of vascular disease.

1. Cystathionine β-synthase deficiency. Homozygous CBS mutations are the most common cause of homocystinuria. Mutations of the CBS gene result in an enzyme with decreased

TABLE 2. Relationship of mutations in enzymes in H(e) metabolism to enzyme function, plasma H(e), and risk of vascular disease

Mutation	Mutation status	Homocysteine levels	Risk of vascular disease
CBS (homo)	Functional	Elevated	Increased
CBS (hetero)	Functional	Normal to elevated	Not increased
G1330A	Functional	Elevated[a]	Unknown[a]
T833C/68-bp ins	Neutral	Normal	Not increased
MTHFR (TS-homo)	Functional	Elevated	Increased
MTHFR (ts-hetero)	Neutral	Normal	Unknown
MTHFR (C677T, homo)	Functional[b]	Normal[b]	Not increased[b]
MTHFR (C677T, hetero)	Neutral	Normal	Not increased
Methionine synthase (A2756G transition)	Neutral	Normal	Not increased
Cobalamine coenzyme synthesis enzymes	Functional	Elevated	Increased

[a] Data from a single case report, so cannot generalize conclusions.
[b] Data from reported studies that have evaluated both genotype and vitamin status show that in folate-replete patients the risk of coronary artery disease and ischemic stroke is not increased. Some authors have suggested this mutation may increase the folate requirement. For this reason the mutation is listed as functional. In patients with low to low normal folate levels, H(e) levels are increased.

affinity for any of its substrates: pyridoxal phosphate, serine, or H(e). Heterozygotes have been found to have variable, but less than 50%, of CBS activity (19). In addition, activity of the mutated CBS enzyme varies as a function of H(e) concentration, possibly due to steric abnormalities in the hybrid normal-mutant molecule. Furthermore, many heterozygotes have normal fasting H(e) levels, and an abnormal MLT does not necessarily imply a heterozygous state. Advances in molecular medicine, however, have now made it easier to assign a heterozygous state to an individual. As a result, CBS genotype is difficult to predict from enzyme activity and emphasizes the need for CBS genotyping in studies correlating CBS mutations with H(e) levels and risk of vascular disease (45).

The CBS gene has been assigned to the subtelomeric region of band 21q22 (46). CBS deficiency is inherited in an autosomal recessive pattern, resulting in homozygous (homocystinuria) and heterozygous (hyperhomocysteinemia) carriers (16). Sequencing of the cDNA for the CBS gene has to date identified 17 mutations (47, 48). Tsai *et al.* (49) have characterized three of the more common mutations as either a $G_{919}A$, a $T_{833}C$, or a $C_{341}T$ transition. The first two mutations account for 50% of affected CBS alleles (50). Different populations demonstrate differing mutation frequencies, with the $G_{919}A$ transition occurring in 70% of an Irish cohort while the $T_{833}C$ transition occurred in 50% of a Dutch population (1). Tsai *et al.* (45) have estimated that the heterozygote frequency for a CBS point mutation is 1/20,000 to 1/200,000, and that 30–40% of individuals with premature vascular disease are heterozygous for CBS point mutations.

An additional mutation described by Sebastio *et al.* (51) involves a 68-bp insertion in the coding region of exon 8 of the CBS gene. This insertion mutation, which creates an alternate splice site at the intron 7-exon 8 border, has only been reported in combination with the $T_{833}C$ missense mutation; the latter is located in *cis* 10 bp upstream of the insertion (46, 49–53). In addition, the insertion sequence contains a premature stop codon; however, Tsai *et al.* (52) reported finding only normal size RNA, implying either that splicing did not introduce the premature stop codon, or that the truncated RNA was not detectable. This insertion results in a benign mutation because the $T_{833}C$ missense mutation is eliminated through alternate splicing at the intron 7-exon 8 border (within the insertion there is no substitution of the 833 nucleotide). The prevalence of this mutation varies with the population studied. Tsai *et al.* (52) reported a prevalence of 11.7% in a control population (heterozygotes); the double mutation has been reported to occur in 25.8% of Northern Italians.

Another novel point mutation has been described by Kluijtmans *et al.* (54) in a partially vitamin B_6-responsive homocystinuric patient. This mutation was a G1330A transition and was unique in that it abrogated CBS responsiveness to SAM. Thus, in contradistinction to the other 17 identified point mutations that result in an altered protein that attenuates the catalytic activity of CBS, this mutation interferes with the regulatory domain of the CBS protein. (54)

2. MTHFR deficiency. MTHFR catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (reaction 3a, Fig. 1). The gene is located on chromosome 1p36.3; 10en different mutations for the MTHFR gene have been identified from isolated cDNA (55). Nine of these mutations result in thermostabile mutations. The two most common of these mutations are a $C_{559}T$ transition, which converts an arginine codon to a termination codon, and a $G_{482}A$ transition, which converts an arginine to a glutamine residue (55). Another mutation in the MTHFR gene, a $C_{677}T$ transition, results in a thermolabile variant (56–58) of the enzyme. This autosomal recessive mutation creates a *Hin*fI restriction site and substitutes an alanine for a valine residue in the MTHFR protein (57). The allele frequency of this polymorphism varies slightly with the population studied, but approximately 8% of studied populations are homozygous for this autosomal recessive polymorphism. The homozygous thermolabile MTHFR polymorphism is characterized by an enzyme activity of about 30% of normal. Heat inactivation at 46 C distinguishes the mutant from the normal MTHFR enzyme. The enzyme is considered thermolabile when there is less than 20% residual activity after heating to 46 C. HH(e) does not occur in individuals heterozygous for this polymorphism and appears only to occur in homozygotes who are concomitantly folate deficient (2, 18, 56–62). Some data suggest that

homozygotes for this mutation may actually have a higher folate requirement (59–61).

3. Methionine synthase. Methionine synthase, localized to chromosome 1q42.3–43 (63), catalyzes the transfer of a methyl group from 5-methyltetrahydrofolate to H(e) via the intermediary methyl-B12. Methionine synthase contains one common polymorphism that results in a A2756G transition. This polymorphism does not correlate with H(e) level and does not appear to be a risk factor for vascular occlusive disease, nor neural tube defects (64). Other rare clinical conditions have reported functional methionine synthase deficiency. These have resulted from mutations in cobalamine coenzyme synthesis that has resulted in abnormal methyl B-12 production, a cosubstrate in the methionine synthase reaction (44, 64–69). No discrete mutations of methionine synthase itself that are associated with hyperhomocysteinemia have been described.

4. Does MTHFR polymorphism increase the risk of MI? Data conflict over the risk of vascular occlusive disease and hyperhomocysteinemia in patients with the homozygous thermolabile polymorphism for MTHFR (1). In contrast, no increased risk for vascular occlusive disease or hyperhomocysteinemia is present in heterozygotes for this polymorphism. Kluijtmans *et al.* (1) screened 60 cardiovascular patients and 111 controls and found that 15% of the cardiovascular patients *vs.* 5% of controls were homozygous for the thermolabile MTHFR polymorphism (1). This translated into a 3-fold risk of premature cardiovascular disease for the homozygous polymorphism. Kluijtmans and coworkers did not evaluate for folate levels in their study. In contrast, Ma *et al.* (19) in the Physicians Health Study reported that the thermolabile MTHFR polymorphism in homozygotes was associated with hyperhomocysteinemia only if folate levels were concomitantly low (19). An increased risk for MI, solely on the basis of the homozygous polymorphism, was not found.

In another study by Legnani *et al.* (18), the presence of the homozygous polymorphism did not increase the risk of thrombosis over control patients (18). Christensen *et al.* (62) showed that the incidence of the homozygous polymorphism and a normal genotype was not different between patients with coronary artery disease and healthy controls, but patients with the homozygous polymorphism did have higher H(e) levels if the serum folate levels were below the median value. Jacques *et al.* (61) reported that homozygotes for the polymorphism had H(e) levels 24% greater than those with normal genotype if the serum folate levels were $<$15.4 nmol/liter. Malinow *et al.* (59) suggested that homozygotes with the polymorphism may have an increased folate requirement, and Ali *et al.* (60) reported that serum folate levels are lower in individuals homozygous for the MTHFR polymorphism. The recent large prospective study by Folsom *et al.* (12) provides additional evidence against the MTHFR mutation being associated with cardiovascular disease (see below for details).

Taken together, these studies suggest that homozygotes may have an increased folate requirement, and that in the presence of normal folate levels homozygotes are not at increased risk for hyperhomocysteinemia or vascular occlusive disease.

C. Nutritional

1. Folate. Plasma H(e) is thus a sensitive biomarker of folate deficiency. Lewis *et al.* (70) demonstrated that in subjects with plasma folate concentrations above 15 nmol/liter the H(e) concentration is on a low, normal plateau. At lower levels of plasma folate, the plasma H(e) concentration increases steadily (38). Rimm *et al.* (71) reported in a large prospective study of women that the 20% who had the highest consumption of folate (almost all of whom consumed multivitamin tablets) had significantly less cardiovascular disease than the lowest 20% (very few of whom consumed multivitamins). However, there have been no randomized control trials of the effect of folic acid alone on cardiovascular disease.

There is controversy about the exact amount of supplemental folic acid that is required to reduce plasma H(e). Shimakawa *et al.* (72) reported that people who use multivitamin supplements have significantly lower plasma H(e) concentrations than nonusers. The RDA for folate in the United States is 200 μg/day. It has been suggested that an intake of 400 μg of folic acid above the dietary level will prevent birth defects. Such an increased supplementation will also significantly decrease plasma H(e) concentrations in most of the population (32).

Recently, Malinow *et al.* (73) reported that breakfast cereal fortified with 400 μg of folic acid was adequate in lowering plasma H(e) by more than 100 μg. Increasing the dose of folate supplementation to 600 μg had very little additional effect.

Schorah *et al.* (74) carried out a randomized double-blind placebo-controlled study in 119 healthy volunteers, whose intake of fortified or supplemental folic acid was low (74). Volunteers were randomized to receive unfortified cereals, or cereals fortified with 200 μg of folic acid per portion, with or without other vitamins, for up to 24 weeks. There were no significant changes in plasma H(e) in those eating unfortified cereals. Folic acid fortification of cereals led to significant increases in serum folate (66%), and red cell folate (24%), and a decrease in plasma H(e) (10%) (74). There were no changes in vitamin B_{12} or cysteine. The H(e) decrease was primarily seen in those who initially had the highest plasma H(e) or the lowest serum folate. Thus, if H(e) is found to be a causative risk factor in occlusive vascular disease, food fortification with physiological levels of folic acid should have a significant impact on the prevalence of the disease in the general population.

de Bree *et al.* (75) evaluated possible inconsistencies between recommended, actual, and desired folate intake in European adult populations. They concluded that in Europe, mean dietary folate intake in adults is 291 μg/day (range 197–326) for men and 247 μg/day (range 168–320) for women. The recommended intakes vary between 200–300 μg/day (men) and 170–300 μg/day (women–with higher recommended intakes during pregnancy). The mean dietary folate intake in Europe is in line with recommendations, but the desired dietary intake of more than 350 μg/day to pre-

vent an increase in plasma H(e) levels is only reached by a small part of studied European populations (76).

A meta-analysis of randomized trials with folic acid (76) reported that supplementation of the typical Western diet with 0.5–5 mg folic acid along with 0.5 mg vitamin B_{12} would be expected to reduce plasma H(e) concentrations by one fourth to one third (*e.g.*, from about 12 μmol/liter to 8 to 9 μmol/liter). Whether such a "population approach" to prevention of HH(e) and associated cardiovascular disease will be effective requires further investigation.

2. Vitamins B_6 and B_{12}. It is well recognized that pyridoxal-5′-phosphate, the active form of vitamin B_6 pyridoxine, is an important cofactor in the conversion of H(e) to cystathionine. The enzyme involved in this reaction is CBS. Because vitamin B_6 is not involved in remethylation, pyridoxine deficiency will only result in HH(e) when the transsulfuration pathway is activated such as after a methionine load. Thus, the positive MLT that was designed to detect heterozygous CBS defects may result from vitamin B_6 deficiency. Rats fed vitamin B_6-deficient diets for 4 weeks develop HH(e) after a methionine load (77). However, clinical studies have demonstrated a lack of a relationship between plasma H(e) and vitamin B_6 status. In human subjects made B_6 deficient by diet (78), fasting plasma H(e) levels remain normal. In patients with asthma treated with theophylline (a vitamin B_6 antagonist), plasma H(e) was significantly higher after a methionine load when compared with controls (79). This abnormality was corrected by treatment with pyridoxine supplementation for 6 weeks. Thus, pyridoxine deficiency should be excluded in patients who have HH(e) after a methionine load – a common abnormality in patients with premature cardiovascular disease.

The importance of vitamin B_{12} in the remethylation of H(e) to methionine is well recognized, and HH(e) is a feature of vitamin B_{12} deficiency (19, 39). However, the relationship between vitamin B12 intake/plasma levels and HH(e)-related cardiovascular disease is less well defined. In the Framingham study, plasma H(e) exhibited a strong inverse association with plasma folate but a weaker association with plasma vitamin B_{12}. Subjects in the lowest decile of plasma B_{12} had significantly higher plasma H(e) when compared with those in the highest decile (80). Homocysteine was also inversely associated with intakes of folate and vitamin B_6, but not vitamin B_{12}. Many of the case control studies of HH(e) in patients with vascular disease have excluded subjects with vitamin B_{12} deficiency. As discussed in the treatment section below, treatment of patients with HH(e) with vitamin B_{12} seems to have very little impact on plasma H(e).

D. Hormones and H(e) metabolism

Studies have been done to clarify the role of hormonal changes in the regulation of H(e) metabolism and in causing HH(e). As indicated above, genetic abnormalities do not fully explain the relatively high prevalence of HH(e) in patients with vascular disease. This is particularly true of post methionine HH(e), which is not often associated with genetic mutations of the CBS gene. Dudman *et al.* (81) suggested that depressed CBS activity in several of their patients was associated with causes other than abnormal CBS protein. A reasonable alternative explanation for the depressed CBS activity would be that it was metabolically down-regulated. Such down-regulation of CBS as well as other enzymes in H(e) metabolism may be mediated by hormonal changes.

1. Estrogen. There is some data to suggest that estrogen has an effect on H(e) metabolism, although the mechanism involved is not clear. Plasma H(e) in pregnant women decreases to almost half that in nonpregnant women (82, 83), suggesting a possible effect of estrogen, although other factors such as increased vitamin intake obviously play a role.

Estrogen may have an effect on the activity of some of the enzymes in H(e) metabolism (84), although further studies are needed to confirm this effect.

Several investigators have examined the effect of menopausal status on plasma H(e). Wouters *et al.* (85) measured fasting and postmethionine plasma H(e) concentrations in premenopausal and postmenopausal healthy women without a history of vascular disease. Fasting and postmethionine plasma H(e) was significantly higher in postmenopausal women as compared with premenopausal women. The difference appears to be too large to be explained as an effect of age alone [the increase in plasma H(e) over a decade of life is modest] and is more likely to be related to hormonal status. In premenopausal women, postmethionine plasma H(e) was negatively and significantly correlated to serum 17β-estradiol. Andersson *et al.* (86) reported that levels of fasting and postload plasma H(e) in premenopausal women did not differ from those of men of similar age. In contrast, in postmenopausal women, the level of fasting plasma H(e) was actually lower than that of men of similar age. This was because, in that study, fasting values in men increased with age and was associated with a decrease in serum vitamin B_{12}, folate, and pyridoxal 5-phosphate.

The rise in H(e) levels after menopause may partly explain the sharp rise in cardiovascular disease that occurs in this age group, and its attenuation by hormone replacement therapy (HRT). However, many cardiovascular risk factors improve with estrogen and further investigation is required to determine whether the lower incidence of vascular disease in premenopausal women and in women taking HRT may be related to the lower concentrations of plasma.

In a prospective study, van der Mooren *et al.* (87) measured fasting serum H(e) during HRT in postmenopausal women. The mean serum H(e) decreased by approximately 11% with a greater decrease (17%) in those women who had a high H(e) level before treatment with very little change in those who had a low level. There are well recognized mechanisms to explain the beneficial effect of HRT on cardiovascular risk, including effects on lipids and fibrinolysis. However, it is possible that a lowering of plasma H(e) also contributes to this benefit.

Oral contraceptive agents, in contrast to HRT, do not affect biochemical folate indices and H(e) concentrations in young women (88).

Tamoxifen, an estrogen antagonist with partial agonist activity, decreased plasma H(e) by a mean of 30% after 9–12 months treatment in postmenopausal women with breast cancer (89). These changes were independent of the tumor

burden. These data, in combination with the effect of estrogen, suggest that there is an estrogen receptor-mediated H(e)-lowering effect. In addition, there may be an indirect effect related to a modest elevation in plasma folate concentrations with tamoxifen (90).

2. Testosterone. Zmuda *et al.* (91) studied the effect of supraphysiological doses of testosterone on fasting H(e) in normal male weight lifters. Plasma H(e) levels were not significantly altered where testosterone was given alone or together with testolactone. It is likely, therefore, that short-term, high-dose testosterone administration does not affect fasting plasma H(e) levels in normal men. However, the study of Zmuda *et al.* has limitations in that only fasting plasma H(e) was measured and, therefore, the authors may have underestimated an effect of testosterone on postmethionine H(e).

Since testosterone has a negligible effect on plasma H(e) concentrations, an alternative explanation is needed for the difference in plasma H(e) concentrations between men and women. Another possible explanation for this gender difference is that creatine-creatinine production is directly coupled to *S*-adenosylhomocysteine generation from SAM (89). Lean body mass, muscle mass, amino acid turnover, and creatine-creatinine production tend to be higher in men than in women, all of which may explain in part the higher plasma H(e) in men. Plasma H(e) concentrations correlate directly with serum creatinine concentrations in men and women (90). In fact, Brattstrom *et al.* (92) demonstrated that the gender difference in plasma H(e) disappeared when men and women were matched for serum creatinine concentrations.

In male-to-female transsexuals, plasma H(e) decreased significantly while it increased in female-to-male transsexuals (93). Thus, the difference in sex steroid milieu between men and women may be an important factor in determining the sex difference between men and women in plasma H(e) levels. Interpretation of these data, however, is complex because of the major changes in sex hormone status occurring in transsexuals. In this context, however, it may be relevant that when inbred adult male rats were treated with estrogen, the plasma H(e) concentration fell by approximately 30%, indicating a direct effect of estrogen on lowering plasma H(e) (93). Plasma H(e) concentrations are also lowered in male rats treated with cortisol, estradiol, or a combination of both (94).

3. Thyroid hormones. An elevated plasma H(e) in hypothyroidism has previously been reported (95). In a recent study, Nedrebo *et al.* (96) demonstrated that plasma H(e) was significantly higher in patients with hypothyroidism (96). In contrast, plasma H(e) in hyperthyroid patients did not differ significantly from that of controls (96). Thus, HH(e) may exacerbate the increased risk of cardiovascular disease in hypothyroidism that is traditionally attributed to lipid changes.

V. Homocysteine and Diabetes Mellitus (DM)

Macrovascular disease is highly prevalent is patients with type 2 diabetes, has an early onset, and progresses at a more rapid rate than in patients without diabetes. While traditional cardiovascular risk factors, such as hypertension and dyslipidemia, are more common in patients with type 2 diabetes, they do not fully explain this acceleration in macrovascular disease (97). Recent data suggesting that H(e) may be a risk factor for cardiovascular disease in this population are, therefore, important (98–100).

Munshi *et al.* (98) reported that HH(e) after a methionine load occurred in approximately 40% of patients with type 2 DM who had macrovascular disease, but was normal in patients with insulin-dependent diabetes (type 1 DM). Patients with overt nephropathy had been excluded from that study, and only patients under the age of 60 were studied. In contrast, Araki *et al.* (101) found that fasting plasma H(e) was elevated in some patients with type 2 DM, and this abnormality was corrected with vitamin B_{12} injections (101). It is possible that these investigators had identified, by chance, patients with concomitant diabetes and B_{12} deficiency. In a population-based study of HH(e) and the risk of cardiovascular disease, 631 patients were stratified according to age, sex, and glucose tolerance (100). The authors also investigated the combined effect of HH(e) and DM with regard to cardiovascular disease. Fasting HH(e) was seen in 25.8% of individuals. After adjustment for age, sex, hypertension, hypercholesterolemia, diabetes, and smoking, the odds ratios (ORs; 95% confidence intervals) per 5 μmol/liter increment in H(e) were 1.44 (1.10–1.87) for peripheral arterial, 1.25 (1.03–1.51) for coronary artery, 1.24 (0.97–1.58) for cerebrovascular, and 1.39 (1.15–1.68) for any cardiovascular disease. After stratification by glucose tolerance category and adjustment for the classic risk factors and serum creatinine, the ORs per 5 μmol/liter increment in H(e) for any cardiovascular disease were 1.38 (1.03–1.85) in normal glucose tolerance, 1.55 (1.01–2.38) in impaired glucose tolerance, and 2.33 (1.11–4.90) in non-insulin-dependent diabetes mellitus ($P = 0.07$ for interaction)(100). Thus, the magnitude of the association between HH(e) and cardiovascular disease is stronger (1.6-fold) for patients with type 2 diabetes than in nondiabetic subjects. Subjects in that study were 50- to 75 yr old, and these findings may relate to an interaction of age, insulin resistance, and diabetes on both H(e) metabolism and cardiovascular disease.

Type 2 DM and HH(e) are both associated with increased lipid peroxidation (oxidative stress) (102, 103). In a study to determine whether the coexistence of elevated H(e) levels stimulate oxidative stress further than that caused by diabetes alone, plasma concentrations of thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation, were measured in patients with type 2 DM. Plasma TBARS concentrations were elevated in diabetics with vascular disease. The additional presence of hyperhomocysteinemia was not associated with a further increase in plasma TBARS concentrations (104). Thus, diabetes maximally stimulates oxidative stress, and any further acceleration of vascular disease in patients who have coexistent hyperhomocysteinemia is mediated through mechanisms other than lipid peroxidation.

Other studies of patients with type 1 DM have confirmed that plasma H(e) levels are normal early in the course of the disease. However, Hultberg *et al.* (105) reported that basal plasma H(e) concentrations were higher in patients who developed nephropathy and had an elevated plasma creat-

inine. These workers recently demonstrated that diabetic patients with the lowest age at onset and poorest metabolic control were most prone to a rapid increase in plasma H(e) (106). They concluded that this increase in plasma H(e) could at least partially be explained by marginal deficiency of blood folate concentrations.

Hofmann *et al.* (107) reported that plasma H(e) levels, both fasting and after a methionine load, were elevated in patients with type 1 DM who had microalbuminuria and were higher still in patients who had overt proteinuria. The patients with type 1 DM and HH(e) also had higher plasma thrombomodulin (TM) levels (indicating endothelial cell damage) and a higher prevalence of late diabetic complications including macrovascular disease.

These workers also reported a significant relationship between plasma H(e) concentrations and urinary albumin excretion rate (Fig. 3), as well as a significant relationship between plasma H(e) and plasma TM (Fig. 4). Thus, HH(e) represents an additional cardiovascular risk factor in patients with microalbuminuria, perhaps contributing to the enhanced risk of cardiovascular disease in this subpopulation of people with diabetes.

Hofmann *et al.* also demonstrated an interaction of H(e) and advanced glycation end products. In human umbilical vein endothelial cells in culture, there was an increased release of TM only when AGE-albumin was added before H(e), indicating a synergistic interaction between advanced glycation end products and H(e), which might contribute to the cardiovascular complications in patients with diabetes (Fig. 5). However, the concentrations of H(e) used in the experiment was more than 100 times that found in plasma in patients with diabetes and HH(e).

The data of Hofmann *et al.* are limited by the fact that there was a much higher prevalence of hypertension in the hyperhomocysteinemic group than in those with normal H(e). It was, therefore, suggested that these subjects had already developed endothelial damage as a result of their hypertension (108). Nevertheless, the possibility remains that renal MTHFR activity may be impaired even in the early stages of diabetic nephropathy, *i.e.*, microalbuminuria (109).

Three recent reports have confirmed the association between plasma H(e) and albumin excretion rate in patients with DM. Chico *et al.* (110) measured fasting plasma H(e) in 165 diabetic patients and control subjects. Patients with type 2 DM had higher plasma H(e) than controls, whereas patients with type 1 DM did not. Univariate correlations and multiple regression analysis showed albumin excretion rate to be strongly related to plasma H(e) (110). In addition, patients with type 2 DM with hypertension had higher plasma H(e) than patients without hypertension (110). This finding may have been related to the fact that the patients with hypertension had more severe biochemical markers of nephropathy, with a higher albumin excretion rate.

Lanfredini *et al.* (111) recently studied the relationship between homocyst(e)inemia and microalbuminuria in non-insulin-dependent diabetes mellitus (NIDDM) patients. There was a significant correlation between urinary albumin excretion and fasting and postmethionine load plasma H(e) in NIDDM patients. Microalbuminuric NIDDM patients had higher fasting plasma H(e) than normoalbuminuric patients. These investigators also reported that patients with NIDDM and HH(e) had higher diastolic and mean arterial blood pressure than those with a normal plasma H(e). There was a significant correlation between plasma folate and mean arterial pressure (112).

Hoogeven *et al.* (113) recently reported a relationship between serum H(e) level, protein intake, and risk of microalbuminuria. In a population-based study of 680 subjects stratified according to age, sex, and glucose tolerance, serum total H(e) was positively associated with the presence of microalbuminuria independent of other risk factors including diabetes, hypertension, protein intake, and renal function. For each 5 μmol/liter increase in serum H(e), the risk of microalbuminuria being present increased by about 30% (114). The authors suggested that hyperhomocysteinemia may partly explain the link between microalbuminuria and the increased risk of cardiovascular disease. However, it is important to recognize that microalbuminuria is associated with several other cardiovascular risk factors, all of which could contribute to increased cardiovascular disease. Nevertheless, in a recent study, Stehouwer *et al.* (114) demonstrated that plasma H(e) predicts mortality in patients with NIDDM, with or without albuminuria. Further investigation is required to determine the exact role that HH(e) plays in worsening cardiovascular disease in patients with microalbuminuria and overt proteinuria.

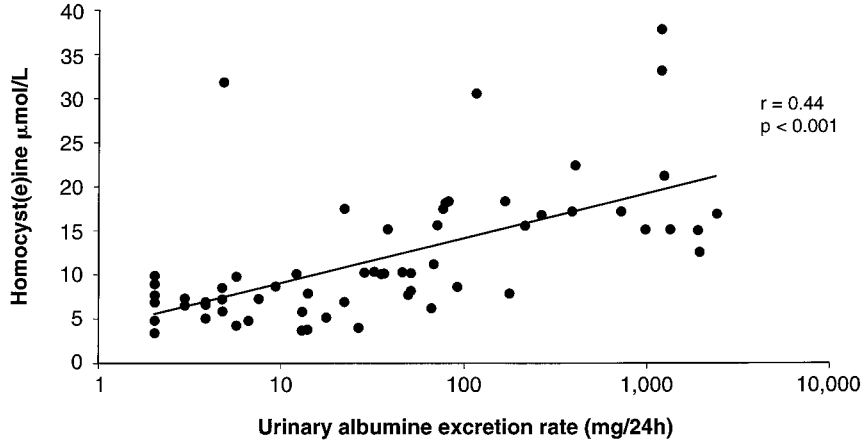

FIG. 3. Relationship between plasma H(e) concentrations and urinary albumin excretion rate. [Reprinted with permission from M.A. Hofmann *et al.*: *Diabetes Care* 21:841–848, 1998 (107). © American Diabetes Association.]

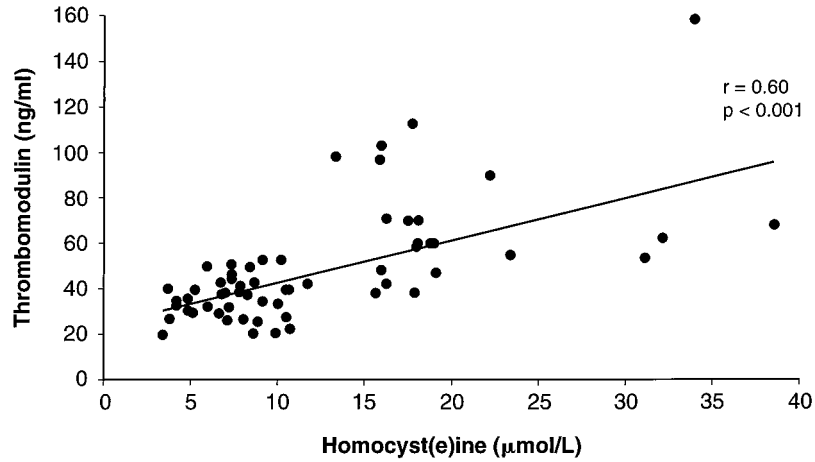

FIG. 4. Relationship between plasma H(e) and plasma TM in patients with type 1 diabetes. [Reprinted with permission from M.A. Hofmann *et al.*: *Diabetes Care* 21:841–848, 1998 (107). © American Diabetes Association.]

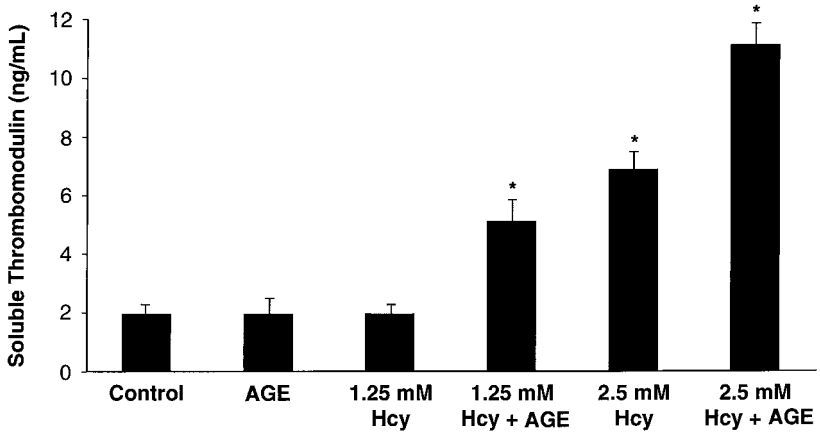

FIG. 5. Effect of the addition of H(e) and HH(e) on human umbilical vein endothelial cells in culture. The release of TM into culture medium has been measured. [Reprinted with permission from M.A. Hofmann *et al.*: *Diabetes Care* 21: 841–848, 1998 (107). © American Diabetes Association.]

A. Hyperhomocysteinemia, renal failure, and diabetic nephropathy

The effect of renal disease on H(e) metabolism has been comprehensively reviewed (115). This subject is of considerable importance to endocrinologists and nephrologists who treat patients with diabetes, as they are at high risk of developing renal failure. Studies to determine whether the treatment of HH(e) in patients with renal insufficiency will reduce morbidity and mortality are thus of considerable importance to physicians treating patients with diabetes.

Recognition of the importance of renal impairment and proteinuria in determining plasma H(e) is vital to the clinician in interpretation of laboratory results. While some of this elevation may be due to decreased clearance of H(e), other mechanisms may also be responsible. Intact kidneys have considerable H(e)-metabolizing capacity (116), impairment of which may be an important determinant of the marked HH(e) frequently observed in end-stage renal disease. High-dose multiple vitamin treatment has been shown to lower plasma H(e) in dialysis patients, although levels remain elevated in many patients (117). Hyperhomocysteinemia has also been demonstrated after successful renal transplantation (118) and may be exacerbated by cyclosporin (119).

B. Effect of glucose and insulin on H(e) metabolism

It is well recognized that insulin has important effects on protein and amino acid metabolism. However, its effect on H(e) metabolism has not been well studied.

During a hyperinsulinemic euglycemic clamp, the plasma H(e) response to acute hyperinsulinemia was heterogenous (120). Plasma H(e) levels fell by approximately 20% in normal subjects but did not do so in insulin-resistant patients with type 2 DM (Fig. 5) (121). These data suggest that resistance to the effects of insulin on glucose disposal may be associated with resistance to the suppressive effect of insulin on H(e) levels in patients with type 2 DM. Such a resistance to insulin's effect on H(e) may contribute to the increased cardiovascular disease associated with the insulin resistance syndrome and type 2 DM (121).

It is well recognized that insulin decreases plasma methionine, the methionine being incorporated into newly synthesized protein (122). Plasma amino acid concentrations, including methionine, fall significantly after an oral glucose load and the subsequent rise in endogenous plasma insulin (123). However, in patients with diabetes, this fall in amino acids does not occur, indicating a possible resistance to insulin's effect on amino acids in diabetics (123).

The insulin-induced fall in plasma methionine concentra-

tions may be mediated through increased tissue uptake of methionine or metabolism via the transsulfuration pathway resulting in increased levels of H(e). Intracellular and plasma H(e) levels may then rise, particularly if a defect in CBS action exists. Further study is required to determine the effect of insulin on SAM, the key determinant of the relative activity of the transmethylation and transsulfuration pathways (see above).

A recent study has demonstrated an inverse relationship between plasma H(e) and insulin sensitivity in women with preeclampsia (124). However, because of the multiple abnormalities in metabolism and endothelial function present in preeclampsia, it is impossible to determine whether a cause-effect relationship exists between the two variables. Further work is needed in other insulin-resistant states to determine whether insulin resistance causes HH(e).

To examine the effects of hyperinsulinemia induced by a high-fat sucrose (HFS) diet on H(e) metabolism, we measured hepatic mRNA and activity of two key enzymes involved in this metabolic pathway: MTHFR and CBS, in an insulin-resistant rat model (125). Fischer rats made insulin resistant by a HFS diet were examined at 6 months and 2 yr of age and compared with control rats fed a low-fat, complex-carbohydrate (LFCC) diet. At the end of 6 months, the HFS-fed rats were heavier than the LFCC rats and had hyperinsulinemia. The plasma H(e) concentrations were elevated in the HFS-fed rats (10.77 ± 0.9 *vs.* 6.89 ± 0.34 μmol/liter; $P < 0.01$). Hepatic CBS mRNA and enzyme activity was significantly lower in the HFS group compared with control. In contrast, hepatic MTHFR enzyme activity and mRNA levels were significantly elevated in the HFS group compared with control. There were significant positive correlations between plasma H(e) and fasting plasma, body weight, and MTHFR activity. There were significant negative correlations between plasma insulin and CBS activity and between CBS and MTHFR activities. The latter inverse relationship supports the hypothesis of Selhub and Miller (21) (Fig. 2) and suggests that insulin's effect on H(e) metabolism may be mediated through SAM. In conclusion, HFS feeding leads to hyperinsulinemia, which may be associated with hyperhomocysteinemia, secondary to down-regulation of CBS message and activity, and may thus contribute to the accelerated macrovascular disease associated with type 2 diabetes. However, we wish to emphasize that the above study was carried out in rodents, and the conclusions may not be applicable to humans.

The effect of drug treatment of diabetes on H(e) metabolism has not been well studied. Evidence suggests that insulin and sulfonylurea treatment do not alter plasma H(e) (101). Hoogeveen *et al.* (126) reported that metformin does not increase plasma H(e). However, in a clinical trial of metformin to assess its lipid-lowering effects in nondiabetic patients with coronary artery disease, there was a moderate but significant increase (7.2% at 12 weeks and 13.8% at 40 weeks) in plasma H(e) with metformin treatment. This was associated with a significant fall in serum B_{12} levels (127).

In summary, HH(e) is not uncommon in patients with diabetes and may play a role in the accelerated cardiovascular disease in these patients. The mechanism for this increased prevalence of HH(e) is not clear but data suggest a role for insulin in regulation of plasma H(e) levels and that insulin resistance may lead to HH(e). Tables 3 and 4 summarize current knowledge of the possible interaction between diabetes and HH(e).

VI. Hyperhomocysteinemia and Cholesterol Metabolism

Evidence is now emerging that the risk for vascular disease from HH(e) is independent of any coexistent abnormalities in lipid metabolism. However, the two risk factors often coexist in the same individual and may have an additive effect.

In 482 patients already at high risk for atherosclerotic vascular disease by virtue of hyperlipidemia, 3.7% had high plasma H(e) (128). In hyperlipidemic patients, the relative risk of atherosclerotic events for the 80th percentile of plasma H(e) was 2.8-fold greater than that seen for the 20th percentile. Furthermore, it was possible to reduce H(e) concentrations in this hyperlipidemic population with vitamins, suggesting a possible therapeutic approach for multiple risk factor intervention. Plasma H(e) has been shown to be associated with a parental history of cardiovascular disease in children with familial hypercholesterolemia (129).

An elevated high-density lipoprotein cholesterol (HDL cholesterol) is well accepted as a protective factor against atherosclerosis. However, a high HDL cholesterol is not necessarily protective in the setting of an elevated plasma H(e) (130).

Considerable *in vitro* data exist to suggest that H(e) may interact with an elevated cholesterol by increasing oxidation of LDL. However, this area remains controversial. It has been suggested that H(e) inhibits glutathione peroxidase activity *in vitro* and leads to a reduction in steady state mRNA levels for the intracellular isoform in endothelial cells (131). Glutathione peroxidase is a member of the antioxidant enzyme family that catalyzes the reduction of lipid peroxides (132).

However, *in vitro* data are not supported by clinical *in vivo* studies, perhaps related to imprecisions in measuring oxidant stress *in vivo*, with current technology. As discussed above in the setting of diabetics with vascular disease, plasma TBARS are not elevated above those levels caused by diabetes alone. These data are supported by two other studies. In one, LDL isolated from two patients with homocystinuria showed a similar extent of copper-catalyzed oxidation as LDL from a group of healthy control subjects (133). Cordoba-Porras *et al.* (134) investigated the existence of oxidized LDL and the susceptibility to oxidation of lipoproteins in six patients with homocystinuria. The proportion of electronegative LDL and concentration of TBARS did not differ between patients and controls (134). Thus, more studies are

TABLE 3. Homocysteine and diabetes

Risk factor for vascular disease
Insulin regulates plasma levels
Elevated in type 2 diabetes
Associated with insulin resistance
Increased in type 1 only with microalbuminuria
Associated with elevated thrombomodulin in type 1 with microalbuminuria
Increased with renal impairment

needed on lipoprotein susceptibility to oxidation in patients with HH(e).

VII. Hyperhomocysteinemia in Premature Vascular Disease

It is well recognized that children who have homozygous homocystinuria develop premature and severe vascular disease. These patients are prone to sudden death in young adulthood or even in childhood. We will now review the data suggesting that moderately elevated H(e) levels are a risk factor for occlusive arterial disease.

A. Epidemiological and prospective studies

In the first prospective epidemiological study of H(e) levels as a cardiovascular risk factor, 14,916 male physicians with no prior vascular disease provided plasma samples at baseline and were followed for 5 yr (6). Plasma H(e) levels in 271 physicians who developed a MI were significantly higher than in paired controls. The relative risk for MI in those physicians in the 95th percentile for H(e) levels was 3 times greater than those in the 10th percentile, even after adjustment for other known risk factors for vascular disease (95% confidence interval, 1.3–8.8) (6).

In 1998, Wald *et al.* (3) reported the British United Provident Association study, a prospective nested case-control study of 21,520 men between the ages of 35 and 64. Homocysteine levels were assayed from stored samples in 229 subjects who died of ischemic heart disease, and 1,126 age-matched controls. Homocysteine levels were higher in the group that died of ischemic heart disease than in the controls. For men with serum H(e) levels in the highest quartile, the increased risk was 2.9 (after adjustment for other factors) than for men in the lowest quartile (3).

In a prospective, nested case-control study within the British Regional Heart Study cohort, Perry *et al.* (135) examined the association between serum total H(e) concentration and stroke. Serum was saved from 5,661 men, aged 40–59 yr, randomly selected from general practices. During follow-up of up to 12 yr, there were 141 incident cases of stroke among men with no history of stroke at screening. Serum H(e) was measured in 107 cases and 118 control men (matched for age group and town, without a history of stroke at screening, who did not develop a stroke or MI during follow-up). Serum H(e) concentrations were significantly higher in cases than controls. There was a graded increase in the relative risk of stroke in the second, third, and fourth quarters of the serum H(e) distribution (odds ratios 1.3, 1.9, 2.8; trend, $P = 0.005$) relative to the first. Adjustment for age group, town, social class, body mass index, hypertensive status, cigarette smoking, forced expiratory volume, packed-cell volume, alcohol intake, diabetes, HDL cholesterol, and serum creatinine did not attenuate the association (135). These findings add to the evidence that H(e) is a strong and independent risk factor for stroke.

B. Studies in patients with established vascular disease

Several studies have attempted to establish the prevalence of HH(e) in patients with premature and accelerated vascular disease. Interest in this field was triggered by the first report by Boers *et al.* (17) who found elevated plasma H(e) after a methionine load in 28% of patients with peripheral vascular and cerebrovascular disease (17). Even after adjustment for other risk factors, plasma H(e) has been found to be significantly higher in patients with peripheral vascular disease compared with healthy individuals (136). Elevations in peak H(e) after a methionine load occur in 28–42% of patients with vascular disease but rarely, if ever, in normal subjects (8, 34, 98). It would appear that the MLT delineates the "at risk" population better than basal levels (34).

In a meta analysis of 27 studies of H(e) in atherosclerotic vascular disease, Boushey *et al.* (7) concluded that elevations of H(e) were an independent risk factor for arteriosclerosis. In addition, approximately 10% of the population's coronary artery disease risk appears attributable to H(e). The odds ratio for development of coronary artery disease from increased plasma H(e) at a level of 5 μmol/liter above normal is 1.6 for men and 1.8 for women.

Fermo *et al.* (137) studied patients below the age of 45 with both venous thrombosis and arterial occlusive disease and found moderate HH(e) in 13.1% and 19.2% of patients, respectively. The prevalence of HH(e) was almost twice as high after a methionine load than when based upon fasting levels. Other studies have confirmed the high prevalence of HH(e) in early onset and recurrent venous thrombosis (10).

Compelling recent evidence comes from the multicenter study done in nine European countries (11). In this study, 750 cases of vascular disease were compared with 800 controls of both sexes younger than 60 yr of age. The relative risk for vascular disease in the highest quintile of plasma H(e) was 2.2 when compared with the lower four quintiles. Methionine loading identified an additional 27% of at risk cases. A dose response effect was noted between total plasma H(e) and risk. The risk was similar to and independent of other risk factors including smoking and hyperlipidemia. An elevated plasma H(e) had a multiplicative effect on risk in smokers and subjects with hypertension. Furthermore, subjects taking vitamins appeared to have a substantially lower risk of vascular disease when compared with nonusers of vitamin supplements. This difference was attributed to lower plasma H(e) levels.

A recent study has established the predictive value of plasma H(e) levels in patients with established coronary artery disease (138) in 587 patients with angiographically confirmed coronary artery disease (many of whom underwent bypass surgery and angioplasty). A strong, graded relationship between plasma H(e) levels and overall mortality was found over a 4-yr period. Less than 4% of patients with a plasma H(e) below 9 μmol/liter died, as compared with nearly 25% of those with a plasma H(e) greater than 15 μmol/liter. The relation of H(e) levels to mortality remains strong after adjustment for other potential confounding variables. It is important to emphasize that plasma H(e) levels are a continuous variable in the population studied, and there is no threshold above which the risk for mortality rises.

In another recent case-controlled study an elevated fasting and postload plasma H(e) showed a positive association with risk of severe coronary atherosclerosis (139). This association

existed over a wide range of plasma H(e), without a clear cut-off point below which there was no increased risk.

Hyperhomocysteinemia appears to have its strongest association with carotid artery disease (140). This finding was also highlighted in a cross-sectional analysis of 1,041 elderly subjects in the Framingham Heart Study. A 2-fold increase in the incidence of carotid disease was seen in patients with the highest plasma H(e) concentrations when compared with those with the lowest concentrations (5). Furthermore, plasma concentrations of folate and pyridoxal-5′-phosphate were also inversely associated with carotid artery stenosis. The Atherosclerosis Risk in Communities Study (ARIC) has reported higher plasma H(e) in subjects with carotid intimal-media thickening (an early marker of atherosclerosis) when compared with matched controls who did not have such thickening (141). Atherosclerotic disease in the aorta as assessed by transesophageal echocardiography also correlates with plasma H(e) (142).

Bots *et al.* (143) examined the relationship of H(e) to MI and stroke among older subjects in a nested case-control study of a subset of participants in the Rotterdam Study. One hundred four patients with a MI and 120 with a stroke were compared with 533 control subjects drawn from the study base, who were free of MI and stroke. Nonfasting total H(e) levels were measured. The risk of stroke and MI increased directly with total H(e) (143). The linear coefficient suggested a risk increase by 6–7% for every 1-μmol/liter increase in total H(e). The odds ratios for subjects in the highest quintile of total H(e) level (>18.6 μmol/liter) compared with those with lower H(e) levels were 2.43 (95% confidence interval, 1.11–5.35) for MI and 2.53 (95% confidence interval, 1.19–5.35) for stroke (143). Associations were more pronounced among those with hypertension (143). The study, based on a relatively short follow-up period, provides evidence that among elderly subjects an elevated H(e) level is associated with an increased risk of cardiovascular disease.

C. Negative studies

Not all studies have shown that H(e) is an independent risk factor for coronary artery disease. A prospective study in Finland of 7,424 healthy subjects at baseline showed development of stroke in 265 subjects over a 9-yr period (144). The fact that the affected subjects did not have an elevated serum H(e) level has been attributed to the exceptionally low gene frequency predisposing to hyperhomocysteinemia in Finland. However, the study itself did not assess the frequency of mutations in the enzymes concerned, and therefore this explanation must be regarded as speculative.

In 1997 Verhoef *et al.* (145) reported a prospective, nested case-control study that used baseline samples from the Physicians Health Study. After 9 yr of follow-up, subjects with newly diagnosed angina pectoris, without MI, and with subsequent coronary artery bypass graft surgery were studied. Controls had no clinical diagnosis of coronary artery disease. In this study, total H(e) levels and risk of angina pectoris did not correlate (145). These data contrast with other reports from this group, which show that H(e) levels correlate with the extent of coronary occlusive disease (6) and that folate and B_6 levels are inversely related to the risk of MI (146). The divergent results may be explained because the study subjects from the Physicians Health (Angina) Study are expected to be nutritionally (folate, vitamins B_{12} and B_6) replete (145). Alternatively, H(e) levels are decreased after MI and then increase over several weeks to months (43). Since H(e) levels were measured 2 to 3 months after the cardiac event in the positive studies, the hyperhomocysteinemia may be the result, rather than the cause, of a vascular occlusive event.

In 1997, Evans *et al.* (13) reported the Multiple Risk Factor Intervention Trial (MRFIT). Prospectively obtained, stored serum samples from 712 men (nonfatal MIs or deaths from coronary artery disease) were analyzed for H(e) level. Odds ratios for patients with coronary artery events based upon H(e) levels are quartile 1, 1.00, quartile 2, 1.03, quartile 3, 0.84, and quartile 4, 0.92. Homocysteine was not found to be a risk factor for coronary artery disease in this study (13).

A recent study by Folsom *et al.* (12) has also questioned the relationship between total H(e) and the risk of coronary artery disease. This prospective case-cohort study consisted of 15,792 men and women ages 45–64, with 232 coronary artery disease cases and 537 controls. Of particular interest, there was no association of coronary heart disease with the thermolabile mutation of the MTHFR gene or with three mutations of the CBS gene (12). These findings in a prospective study add uncertainty to conclusions from other studies that H(e), in general, and the enzyme mutation, in particular, are a major independent risk factor for coronary artery disease. After adjustment for age, plasma H(e) was positively associated with coronary arterial disease in women but not in men (12). Similarly coronary artery disease was negatively associated with plasma folate and vitamin supplementation in women only. After correction for other risk factors, only pyridoxal 5′-phosphate plasma levels were associated with the risk of coronary artery disease (12).The finding that pyridoxal 5′-phosphate levels are important in determining the risk of coronary artery disease may be related to the metabolic mechanisms used by these patients to handle hyperhomocysteinemia. The investigators studied only fasting plasma H(e), and the fact that vitamin B_6 appeared to offer independent protection suggests that high postmethionine load H(e) (determined by vitamin B_6, as discussed above) may have been a better variable to study as a risk factor for coronary artery disease than fasting H(e).

In summary, data from many studies support the hypothesis that hyperhomocysteinemia is an independent risk factor for coronary artery disease, as well as other arterial occlusive disease. More recently, data from several studies have questioned whether H(e) *per se* is a risk factor for coronary artery disease. Both positive and negative studies have shown the importance of vitamin levels in study subjects, as well as controlling for other known cardiovascular risk factors, including gender. Indeed, several of the studies indicate that hyperhomocysteinemia is a greater risk factor in women or older subjects in whom vitamin levels may also be lower. The timing of H(e) collection after a vascular occlusive event may also affect the H(e) level. Future studies to clarify the relationship between H(e) and coronary artery disease must therefore be prospective, control for known cardiovascular risk factors, and measure vitamin B_6, B_{12}, and folate levels.

D. Effect of low plasma H(e) on cardiovascular disease

If an elevated plasma H(e) is associated with an increased risk of coronary heart disease, then a low plasma H(e) should lead to a decreased risk of coronary heart disease. However, information on plasma H(e) concentrations in population groups with low coronary artery disease prevalence is limited and conflicting.

One such population is patients with Down's syndrome, a condition that is associated with a very low prevalence of coronary artery disease (147). The gene for CBS is on chromosome 21, and trisomy 21-associated Down's syndrome is associated with a CBS gene dosage of 150% (148) and significantly lower fasting as well as postmethionine plasma H(e) concentrations (149). However, Brattstrom *et al.* (150) failed to find more effective H(e) metabolism in Down's syndrome patients.

Ubbink *et al.* (151) studied a population of South African black subjects living in a rural area who had a low incidence of coronary artery disease, despite a high prevalence of smoking and in whom plasma H(e) concentrations were significantly lower than those in South African whites. They also reported that the distribution of plasma H(e) concentration frequencies was positively skewed; they suggested that this frequency distribution corresponds to that previously reported in populations prone to coronary artery disease (6, 140). While the postmethionine load H(e) fell after vitamin treatment in white subjects, it did not do so in blacks; indicating relative independence from this nutritional cofactor and the possibility of other cofactors or regulators in H(e) metabolism.

In another study attempting to determine the cause of difference in prevalence of coronary artery disease, plasma H(e) concentrations were found to be higher in people in Ireland compared with those in France (152). The prevalence of coronary artery disease and mortality in Ireland is much higher than that in France. Although there are differences between the two populations in conventional risk factors, these do not account for the large interpopulation difference in coronary artery disease. A higher plasma H(e) in the Irish subjects who suffered an MI when compared with that in the French could explain the different rates of coronary heart disease in the two populations. This study also showed that the risk for MI in both populations was graded across the distribution of plasma H(e) and increased in subjects with the highest plasma H(e)(152).

We have recently studied plasma H(e) concentrations in lean subjects with type 2 diabetes in India and have found that plasma H(e) concentrations are lower than those in normal weight and obese diabetic subjects (S. Das and V. Fonseca, unpublished observations). Coronary artery disease is rare in lean subjects in India despite the presence of DM (152). However, the prevalence of diabetes in Indians is higher than that in Caucasians (153). These patients have been found to be insulin resistant despite modest degrees of obesity, and the incidence of coronary artery disease in Indian immigrants to the United Kingdom has been found to be exceedingly high (154). Thus, it would appear that even modest degrees of obesity are associated with a rise in plasma H(e) concentrations in this population when compared with lean subjects. These findings are compatible with our data on HFS feeding in rats described above.

In summary, the prevalence of vascular disease appears to be lower in populations that have lower levels of plasma H(e). However, a changing phenotype, particularly with an increase in obesity, may be associated with an increase in plasma H(e) and a concomitant increase in cardiovascular risk. Whether these variables are causally related needs to be investigated.

VIII. Possible Mechanisms Of Accelerated Vascular Disease in Homocysteinemia

Putative mechanisms of atherothrombosis in hyperhomocysteinemia include endothelial cell injury, endothelial dysfunction, increased vascular smooth muscle cell growth, increased platelet adhesiveness, enhanced LDL oxidation and deposition in the arterial wall, and direct activation of the coagulation cascade. Caution should be used in extrapolating the results of *in vitro* studies, as in many of them the concentrations of H(e) used are much higher than those seen in plasma of patients with HH(e).The vascular changes in hyperhomocysteinemia are likely to be multifactorial (Table 4).

A. Platelet dysfunction

Platelets from patients with HH(e) have increased adhesiveness which is corrected by pyridoxine (155). Treatment with pyridoxine also restores the decreased platelet survival seen in some patients. Homocysteine alters arachidonic acid metabolism in platelets, with increased release of proaggregatory thromboxane A_2 (156). In rats in whom mild HH(e) has been induced by folate deficiency, an acute methionine load enhances platelet aggregation, thromboxane biosynthesis, and macrophage-derived tissue factor activity (157).

B. Coagulation abnormalities

Activation of the coagulation cascade by H(e) may also contribute to vascular disease. Homocysteine activates Factor XII and induces arterial endothelial cell Factor V activation (158, 159). In addition, high concentrations of H(e) may inhibit TM (160, 161). Since the binding of thrombin to TM enhances formation of the anticoagulant-activated protein C and inhibits thrombin activation of fibrinogen, a deficiency of TM enhances fibrin formation. All of these events effectively change the balance between procoagulation/anticoagulation and enhance the risk of thrombosis. In patients with the more severe condition of homocystinuria, there is activation and hyperconsumption of Factor VII, Factor X, and consumption of antithrombin III (161–164). In homocystinuria, levels of coagulation factors are reduced. Markers of activation of coagulation, such as F1+2, are elevated and are correctable with treatment (165). H(e) increases tissue factor activity in a dose-dependent fashion, by increasing the rate of synthesis of tissue factor RNA (166).

C. Effects on the endothelium

Recent investigation has identified the endothelium as a major site of pathological damage caused by HH(e). In par-

TABLE 4. Possible mechanisms contributing to premature atherosclerosis in hyperhomocystinemia

Endothelium
Patchy endothelial cell loss
Decreased viability and function
Free radical generation and lipid peroxidation
Induces tissue factor
Increased vWF, thrombomodulin, and PAI-1
Decreased prostacyclin
Decreased DNA synthesis
Decreased nitric oxide
Decreased vascular reactivity
Platelets
Decreased survival
Increased adhesiveness
Increased aggregation
Increased release of platelet factors that stimulate smooth muscle proliferation, vasoconstriction, and aggregation
Coagulation
Inhibition of cell surface expression of thrombomodulin
Decreased activation of protein C
Decreased activity of antithrombin III, decreased factor VII
Arterial wall
Vascular smooth muscle cell proliferation and hypertrophy
Increased intimal-medial thickness

ticular, the interaction between H(e) and nitric oxide (NO) described below has important clinical implications.

Vascular reactivity (determined by assessing the change in brachial artery diameter during reactive hyperemia – an index of flow-mediated, endothelium-dependent, nitric oxide-mediated vasodilatation) is significantly impaired in elderly subjects with HH(e), compared with control subjects (167). In contrast, the vasodilatation after the administration of sublingual nitroglycerin (endothelium-independent) is normal. On linear regression analysis serum H(e) concentrations emerged as the only significant predictor of flow-mediated vasodilatation. These results indicate that the bioavailability of nitric oxide is decreased in human subjects with HH(e) and is compatible with the findings in animals. Celermajer *et al.* (168) demonstrated that children with homozygous homocystinuria had impaired endothelial function and vascular reactivity. In contrast, endothelial function assessed by similar methodology is preserved in heterozygous adults.

In a primate model, Lentz *et al.* demonstrated that diet-induced HH(e) led to blunted responses of resistant vessels to endothelium-dependent vasodilators and that this effect was accompanied by depressed TM activity and moderately reduced vascular smooth muscle responses to nitroglycerin when vessels were studied *ex vivo* (169). An infusion of H(e) in rats abolishes the endothelium-dependent vasodilation induced by acetylcholine, suggesting that homocysteine's adverse effects are mediated through deficient production of NO (170).

Normal endothelial cells detoxify H(e) by releasing NO, which leads to the formation of *S*-nitroso-homocysteine (171). The formation of S-nitroso-homocysteine attenuates the pathogenicity of H(e) by inhibiting sulfhydryl-dependent generation of free oxygen radicals. This protective action, however, is eventually overcome by chronic exposure of the endothelial cell to HH(e) (103). Homocysteine may also attenuate endothelial production of bioactive NO (103).

The enzymes in H(e) metabolism are present in endothelial cells (172), and H(e) metabolism is active in endothelial cells. In human umbilical vein endothelial cells in culture, H(e) is exported into the culture medium, and this export is decreased by folate in a dose-dependent manner (173).

Endothelial cells from CBS heterozygotes are deficient in CBS and are more susceptible to H(e)-mediated injury (174). Jakubowski has demonstrated that human cells in which H(e) metabolism is deregulated by a mutation in the CBS gene or by the use of antifolate drug produce more H(e) thiolactone than unaffected cells (175). The thiolactone is incorporated into cellular and extracellular proteins and may be more toxic to endothelial cells than H(e).

In response to H(e)-induced toxicity in human endothelial cells, substantial changes in the concentration of intracellular soluble thiols have been observed (176). Large decreases in cellular NAD+ occurred in response to H(e)-induced toxicity, and DNA synthesis was also compromised. Radical scavengers were effective in preventing this H(e) toxicity (176).

Hyperhomocysteinemia after a methionine load in rats is associated with considerable loss of endothelium and degeneration of media cells in the aortic wall. These changes are more pronounced in the spontaneously hypertensive rat than in the normotensive rat (177).

There has been considerable interest in HH(e) causing endothelial damage by increasing free radical production and subsequent lipid peroxidation (178, 179). However, the exact role of free radicals in HH(e)-induced endothelial damage remains unclear.

Anticoagulation and fibrinolysis are also functions of the endothelium that are critical for blood flow. Several studies have been concerned with measuring the endothelial-derived proteins important in these processes. Plasma concentrations of proteins secreted by the endothelium such as TM and von Willebrand factor (vWF) are elevated in HH(e) and serve as surrogate markers of endothelial dysfunction (180). Van den Berg *et al.* assessed endothelial function by measuring plasma concentrations of endothelium-derived proteins such as vWF, TM, and tissue-type plasminogen activator (tPA) (181); vWF and TM were elevated while tPA was normal in patients with HH(e). After treatment with pyridoxine and folic acid, vWF and TM levels decreased and tPA was unchanged. Tissue plasminogen activator inhibitor (PAI-1) antigen has been shown to correlate with total plasma H(e) concentrations and may also be a marker of impaired fibrolytic activity and endothelial function (182). H(e) has also been shown to suppress anticoagulant heparin sulfate expression in cultured porcine aortic endothelial cells and may thus contribute to thrombogenesis (183). Finally, H(e) inhibits cyclooxygenase activity in human endothelial cells, decreasing prostacyclin production (184).

In summary, H(e) metabolism is active in endothelial cells, and endothelial damage with loss of ability to generate adequate NO appears to be the major mediator of vascular disease caused by HH(e). In addition, the endothelial secretion of anticoagulant and fibrinolytic substances is impaired. Depletion of NO results in loss of vasodilatation, generation of reactive oxygen species, inhibition of glutathione peroxidase, proliferation of vascular smooth muscle cells, and suppression of endothelial cell growth. The importance of these

in vitro mechanisms *in vivo,* in a setting of more physiological concentrations of H(e), is unclear. Additional *in vivo* studies that utilize physiological concentrations of H(e), demonstrate a H(e)-specific effect, and evaluate reversibility of endothelial damage with vitamin therapy are needed. In particular, these studies must evaluate whether endothelial damage results from or causes elevated plasma H(e) concentrations.

D. Effects of hyperhomocysteinemia on the arterial wall

Homocysteine appears to have a growth-promoting effect on the arterial wall through a number of mechanisms. Tsai *et al.* (185) studied the effect of H(e) on the growth of vascular smooth muscle cells and endothelial cells. H(e) causes a 25% increase in DNA synthesis in rat aortic smooth muscle cells. In contrast, in human umbilical vein endothelial cells, H(e) leads to a decrease in DNA synthesis in a dose-dependent manner. These findings suggest that H(e) has a growth-promoting effect on vascular smooth muscle cells along with an inhibitory effect on endothelial cell growth. This combination could lead to atherosclerosis (185). Minipigs fed a methionine-rich casein-based diet develop HH(e). These animals developed aberrations in the elastic lamina with hypertrophy of smooth muscle (186).

Rats fed a high H(e) diet have a stimulation of aortic cyclin-dependent kinase at the transcriptional level, with the possible consequence of proliferation of aortic cells (187). Arterial smooth muscle cells cultured in the presence of H(e) grow to a higher density and produce and accumulate collagen at levels significantly above controls (188). Homocysteine also inhibits growth and p21ras methylation in vascular endothelial cells (189). Homocysteine has also been shown to have a weak mitogenic effect on vascular smooth muscle cells and, in addition, enhances the mitogenic response of platelet-derived growth factor (190).

E. Coinheritance of factor V Leiden in homocystinuria

The coinheritance of homocystinuria and factor V Leiden mutation (activated protein C resistance) has been found to have an association with thrombophilia (10). Because only one-third of patients with homocystinuria develop venous or arterial thromboses, a search was made for other contributing factors in patients who developed thrombosis. A mutation in the gene coding for factor V replaces glutamine for arginine at position 506, increasing a patient's risk for thrombosis by altering the first cleavage site involved in the activation of factor V. This suggests that in patients with homocystinemia, the risk of venous or arterial thromboembolic disease may be exacerbated by the presence of other concomitant etiologies of thrombophilia.

IX. Management of Hyperhomocysteinemia

A number of agents are known to decrease plasma H(e) concentrations in patients with both HH(e) and homocystinuria. Some of these, such as folate and pyridoxine, are inexpensive and safe. However, clinical trials are needed to demonstrate their efficacy in preventing or halting the progression of vascular disease.

A. Prevention of hyperhomocysteinemia

The role of folate, B_{12}, and pyridoxine in determining plasma H(e) levels in normal subjects has been discussed above. We have also discussed clinical trials of foods fortified with folate in the prevention of HH(e). It is tempting to speculate that such food fortification will be effective in preventing vascular disease. However, outside the setting of a few clinical trials the impact of increased nutritional supplementation with folic acid, which has been recently recommended (191), on H(e) levels in the general population is unknown and needs to be evaluated.

B. Treatment of hyperhomocysteinemia

There is no consensus on the optimal dosage of vitamins to be used in the treatment of HH(e). Not only are clinical trials necessary to determine whether lowering plasma H(e) will reduce cardiovascular disease, the optimal dose necessary to do so will need to be ascertained. Practicing physicians face a dilemma on prescribing therapy for HH(e) because of media advertising recommending multivitamins and fruit juices, cereals, etc., to reduce cardiovascular disease, when there is very little evidence that such treatments may be effective.

Combination therapy with different vitamins may be necessary to achieve adequate suppression of H(e) levels in many patients. For example, Franken *et al.* treated mild HH(e) patients with vitamin B_6, 250 mg daily, for 6 weeks after which the post load H(e) concentration fell in 56% of patients (192). Further treatment with the addition of folic acid and/or betaine resulted in normalization of H(e) levels in 95% of the remaining patients. In three patients with homocystinuria, Palareti *et al.* (193) demonstrated not only reduction in H(e) levels but also correction of a number of abnormalities in blood coagulation (193). It is important to determine whether correction of coagulation and other vascular risk factors occurs in patients with mild HH(e) after treatment.

Only half of patients with CBS deficiency respond to pyridoxine. This may be because some nonresponders may have folate deficiency (194) (which can block the response to pyridoxine until folate is replenished) (21) or decreased affinity of the mutant enzyme for the cofactor (195). Van den Berg *et al.* (181) have also demonstrated correction of mild HH(e) in a subset of young patients with cardiovascular disease (below the age of 50). Brattström *et al.* (196) demonstrated that pyridoxine, 240 mg/day, plus folic acid, 10 mg/day, reduced fasting H(e) levels by a mean of 53% and a postmethionine load H(e) by a mean of 39%.

Ubbink and colleagues (197, 198) investigated the roles of three vitamins as determinants of plasma H(e) concentrations in a placebo-controlled study. One hundred individuals with high fasting plasma H(e) were enrolled in the trial, which compared treatment with five different treatments: 1) placebo; 2) folic acid, 0.65 mg; 3) pyridoxine, 10 mg; 4) vitamin B_{12}, 0.4 mg; and 5) a combination of all three vitamins.

Plasma samples were assayed 4 and 6 weeks after starting the vitamin therapy. The folic acid lowered plasma H(e) by 42%, but plasma H(e) declined with vitamin B_{12} by only 15%. Pyridoxine had no significant effect, which is not surprising as the main effect of pyridoxine would be on lowering postmethionine plasma H(e) rather than a fasting H(e) (33, 199). The combination of three vitamins did not differ significantly from folic acid alone (197).

Boers *et al.* (200) treated 32 patients with postmethionine load HH(e) with vitamin B_6 250 mg for 6 weeks. A few patients also received folic acid 5 mg daily in addition (if they were folic deficient); 81% of patients responded with the normalization of postload HH(e). Similarly, Brattström *et al.* (196) reported a 26% reduction in postload plasma H(e) between 15 mg daily of vitamin B_6 and further significant reduction of up to 39% when folic acid 10 mg daily was added (196). Dudman *et al.* (33) showed similar results with supplementation of 100 mg of vitamin B_6. There was a further reduction in postload levels using a combination of folic acid, 5 mg daily, and vitamin B_6, 100 mg daily.

van den Berg *et al.* (201) reported that treatment of patients with HH(e) with vitamin B_6, 250 mg, plus folic acid, 5 mg daily for 6 weeks, resulted in normalization of the fasting plasma H(e) in 91% and postload plasma H(e) in 92% of patients. Landgren (43) studied the effect of various doses of folic acid on fasting plasma H(e) after myocardial function. Folic acid at 2.5 mg daily and 10 mg daily were equally effective in lowering fasting H(e) levels with a greater reduction seen in patients who had elevated plasma H(e) at the start of the study (43).

Treatment with vitamin B_{12} does not appear to have a very significant effect on plasma H(e) levels in healthy subjects but may reduce it in some patients with HH(e), particularly those who have low or normal vitamin B_{12} blood levels (198).

In summary, although no consensus exists it seems likely that folic acid, 0.65 mg daily, may be sufficient to lower mild fasting HH(e) significantly. However, in patients who have a MLT and are found to have an elevated postload plasma HH(e), treatment with at least 100 mg daily of vitamin B_6 and 5 mg of folic acid daily is necessary. The dose of vitamin B_6 may need to be increased to 250 mg daily.

1. Betaine. Betaine is a methyl group donor involved in the metabolism of methionine and has been suggested as a possible treatment for HH(e) (202). Betaine lowers plasma H(e) concentration but raises methionine levels, the significance of which is not clear (203, 204). Betaine has been found to be ineffective in lowering H(e) in patients on hemodialysis (115).

The above treatment strategies may not apply to patients with very advanced aggressive vascular disease or patients with chronic renal failure and/or DM, where other factors may elevate H(e) levels. These patients may require much higher doses of vitamin replacement therapy (115).

X. Conclusion

Hyperhomocysteinemia is now well established as a risk factor for cardiovascular disease. HH(e) is common in patients with premature and accelerated vascular disease and is a risk factor for death in patients who have an MI. Genetic abnormalities and nutritional deficiencies explain only a small proportion of the hyperhomocysteinemia associated with vascular disease. Hormonal and metabolic factors such as diabetes, thyroid disease, and estrogen deficiency appear to interact with H(e) metabolism and may thus play a role in the pathogenesis of atherosclerosis seen in patients with these conditions.

Further investigation into the role of hormonal changes in H(e) metabolism may lead to an improvement in our treatment strategies to prevent atherosclerosis. Fortification of food with folate and nutritional supplementation with vitamins lowers plasma H(e). Combination therapy with multiiple vitamins may be nescessary to correct HH(e) in many patients. Clinical trials are needed to determine the optimal doses of vitamins in different clinical settings. Finally, it is important to determine whether such a lowering of plasma H(e) will prevent the onset or progression of cardiovascular disease.

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