Sulfur amino acids and atherosclerosis: a role for excess dietary methionine

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The homocysteine theory of arteriosclerosis received credence when it was shown that after a methionine load, circulating homocysteine–cysteine concentrations were higher in cardiovascular disease patients than in healthy controls. Subsequent studies showing associations between homocysteine and coronary artery disease, stroke and cognitive impairment, relied on small increases in homocysteine concentration unlike the very high homocysteine seen in the rare genetic disorders that lead to homocystinuria and much higher homocysteine levels. Subsequent studies in cell culture, animals, and humans showed that a variety of cardiovascular adverse effects of “high homocysteine” introduced either as a nonphysiological bolus or as a methionine load led to high homocysteine. We fed apolipoprotein E–deficient mice diets designed to achieve three conditions: (1) high methionine intake with normal blood homocysteine, (2) high methionine intake with B vitamin deficiency and hyperhomocysteinemia, and (3) normal methionine intake with both B vitamin deficiency and hyperhomocysteinemia. We found that the mice fed methionine-rich diets had significant atheromatous pathology in the aortic arch even with normal plasma homocysteine levels. Mice fed B vitamin–deficient diets developed severe hyperhomocysteinemia but without any increase in vascular pathology. Our findings suggest that even moderate increases in methionine intake are atherogenic in susceptible mice while high plasma homocysteine is not.

Keywords: methionine; homocysteine; folate; atherogenic; ApoE deficiency

Introduction

Methionine is a sulfur amino acid with essential roles in intermediary metabolism. In addition to functioning as a protein building block, methionine can serve as a precursor for cysteine and glutathione, and is the precursor of both S-adenosylmethionine (SAM), the universal methyl donor for over 100 reactions, and of certain polyamines (Fig. 1). These last two functions render methionine an essential amino acid, even though the mammals are capable of its synthesis. In this synthesis, homocysteine, a product of the SAM-dependent methylation reactions, is methylated to form methionine, a reaction catalyzed by two different enzymes: betaine–homocysteine methyltransferase (EC 2.1.1.5) that locates in the liver and to some extent in the kidney; and 5-methyltetrahydrofolate–homocysteine methyltransferase (EC 2.1.1.13), which is vitamin B12 dependent, is found in all tissues, and is embryonic lethal if its gene is knocked out.1

The relationship between methionine and arteriosclerosis was first pointed out by McCully2 in 1969 who noted the high prevalence of early arteriosclerotic and thromboembolic disease in patients with congenital homocystinuria. Initially, this proposal received little attention. Homocysteine concentrations in these patients with rare congenital defects leading to homocystinuria were uncommonly high and were not deemed a public health risk. However, in 1976, Wilken and Wilken3 published a study showing that after a methionine load concentrations of homocysteine–cysteine
mixed disulfide were significantly higher in 10% of the patients (n = 22) compared to age-matched healthy controls. What was particularly important about this observation was that concentrations of the homocysteine–cysteine mixed disulfide in cardiovascular disease (CVD) patients were far lower (17 μM) than those (≥200 μM) in patients with vitamin B12–related or cystathionine synthase–related congenital disorders. The fact that the homocysteine in CVD patients is only slightly higher than normal nevertheless became the basis for numerous studies in cell cultures, animals, and humans. Most assumed that the slightly higher homocysteine found in patients with CVD and other diseases was at least a partial cause of the disease and that this could be modified by vitamin intake to lower the exposure to homocysteine. For example, studies in which cultured cells were incubated with homocysteine showed a variety of deleterious effects, including inhibition of prostacyclin synthesis, activation of factor V, inhibition of protein C activation, downregulation of thrombomodulin expression, and blocking the binding of tissue plasminogen activator (t-PA)—but not of plasminogen—to endothelial cells.4,5 Other toxic effects attributed to homocysteine include increased platelet adhesion, impaired regulation of endothelium-derived relaxing factor and related nitrogen oxides, induction of tissue factor, suppression of heparin sulfate expression, stimulation of smooth muscle cell proliferation, and oxidation of low-density lipoprotein.

A major limitation of all the above-mentioned observations is that the pathophysiological significance of the experimental exposure of cells to high homocysteine concentrations is indeterminate. Human plasma contains two sulfur-containing amino acids, homocysteine and cysteine, at respective mean normal concentrations of 10 and 240 μmol/L. In mild hyperhomocysteinemia, the levels of homocysteine are on average 30% higher than normal, in contrast to rare, severe cases of homocystinuria, where the levels may be as high as 200–400 μmol/L.6,7 As cysteine appears to be harmless and to bear no relationship to disease, it was inferred that the putative action of homocysteine on the blood vessel wall and the coagulation system is stereospecific, and that it involves the entire molecule and takes place at low concentrations (in the micromolar range). However, these crucial considerations were rarely addressed in studies on mechanism.
The homocysteine concentrations that were typically used (1–10 mmol/L) often exceeded the levels encountered even under the most severe pathological conditions. Thus, the possibility that the observed effects were due to nonspecific reactivity of the sulfhydryl group of the homocysteine molecule could not be ruled out. Indeed, in several studies where other thiol-containing compounds such as cysteine and mercaptoethanol were tested, the effects were similar to these seen with homocysteine (for example, see Ref. 8 and also Refs. 9–12). Thus, it is difficult to know whether such mechanisms might also mediate the increase in relative risk found in human observational studies, where there is a far milder but chronic exposure of the arterial wall to micromolar increments of circulating homocysteine.

In an attempt to examine whether such mechanisms occur in vivo, homocysteine levels can be experimentally raised by dietary interventions in both animal models and in humans by depleting folate, vitamin B12 and vitamin B6; by direct intake of homocysteine; and by an oral methionine load. The methionine load was first introduced to identify subjects who are heterozygotes for cystathionine β-synthase deficiency13,14 and later routinely used to identify hyperhomocysteinemic patients that for some reason or another have higher postload homocysteine levels.15 Importantly, the methionine load has consistently been found to impair vascular function. For example, Bellamy et al.15 showed that a postmethionine load increase in homocysteine from 7.9 to 23 μM was associated with a substantial decrease in flow-mediated brachial artery dilatation (from 0.212 to 0.06 mm). A second study of 20 healthy individuals by Nappo et al.16 has shown that a methionine load was associated with increased homocysteine from 10 to about 23 μM and increased coagulation and circulating adhesion molecule levels (sICAM-1, sVCAM-1, tPA, F1+2, fibrinopeptide, PAI-1, and D-dimer) and impaired vascular response to arginine load. What is interesting in this study is that administration of vitamins E and C with the methionine prevented all the vascular outcomes except for homocysteine, which remained high. This finding raises the possibility that either the vitamins prevent the toxic effects of homocysteine by interfering with its reaction on molecular targets or that homocysteine is not the effector of toxicity. Some studies have shown that oral methionine loading is associated with lower flow-mediated dilatation (FMD),17 while others have suggested that methionine loading is associated with a lower FMD in type 2 diabetes.18 In rats, excess methionine intake was associated with decreased elasticity of aortic lamina and increased thickness of the aortic wall.19 Morita et al.20 have shown that feeding high methionine (1.2–2.0%) or homocysteine to rats exacerbates neoformation after a denuding injury, thereby setting the stage for more rapid restenosis. Folate could ordinarily ameliorate the detrimental effects, but not when rats were fed 2% methionine. Other studies in rats, rabbits, and mini pigs show similar results.21–23 In the most recent study in healthy humans, an oral methionine load induced a significant increase in total plasma homocysteine concentrations and decreased aortic distensibility within 3 hours. These were also associated with a significant increase in Tei index, a measure of myocardial function (P < 0.001) that suggests a worsening compared with baseline values.24

Taken together, these and other studies show that oral methionine loading is undoubtedly associated with adverse outcomes that are closely related to endothelial cell dysfunction and to increased risk of CVD. The question remains, however, whether these effects are the reflection of the increased homocysteine concentration caused by the high level of methionine load, by methionine per se, or by other aspects of its metabolism. According to Ubbink et al.,25 4 h after a methionine load of 100 mg/kg body weight, plasma methionine level reaches about 400 μmol/L compared to about 25–30 μmol/L homocysteine. Many of the studies that employed the methionine loading test in humans or prolonged feeding of high homocysteine in animal models have not controlled for the possibility that it is the high methionine rather than the high homocysteine that produced these effects. Moreover, the theory that circulating homocysteine is directly atherogenic cannot account for the fact that although deleting the cystathionine beta synthase (CBS) and the methylene tetrahydrofolate reductase (MTHFR) genes in mice both cause severe hyperhomocysteinemia, neither produce the vascular pathology seen in human with congenital defects in these genes.26,27 In addition to their effects on circulating homocysteine, both these mutations cause an imbalance of methionine metabolism.
The idea that excess methionine intake is toxic is not new.28 A typical study published in 1970 showed that feeding guinea pigs a diet with 10 mM/kg l-methionine per day rapidly leads to a state characterized by fatty liver, hypoglycemia, and aminoacidemia and later by hypothermia, profound hypoglycemia, and death within 60 hours.29 There was also a substantial decrease in hepatic adenosine triphosphate most likely due to interaction with methionine to form SAM.

Homocysteine is a nonprotein-forming amino acid, which functions as both a product and a substrate of methionine (Fig. 1). Hyperhomocysteinemia can therefore occur either as a result of excessive intake of methionine as in the methionine loading studies where the homocysteine methylation machinery is insufficient to remethylate the large amount of homocysteine produced or in the absence of vitamins, or in those with congenital defect in genes that code for enzymes in the one carbon metabolic pathway.

We took advantage of the biochemical pathways of homocysteine metabolism to assess whether the two types of hyperhomocysteinemia, one caused by B vitamin deficiency and one by excess methionine differ with respect to their effects on atherogenesis.30

For this purpose, we chose the apolipoprotein E (ApoE)–deficient mice.31–33 These mice develop hypercholesterolemia and spontaneous atherosclerotic lesions in the aortic root and branch points in a pattern resembling human atheromas34 that include the accumulation of oxidized lipids34–37 and inflammatory markers.38 Moreover, they develop spontaneous vascular lesions in vivo, and can therefore be used to assess various diets as potential modifiers of the rate and extent of lesion progression.36,39–44

We allocated young male ApoE-deficient mice to receive one of four diets: (1) a control diet formulated with 3.3 g methionine/kg diet, (2) high methionine (7.7 g methionine/kg diet) in combination with B vitamin deficiency (less than 10% of control levels of required B vitamins folate, B12 and B6) (M+B−), (3) B vitamin deficiency with normal methionine (B−), and (4) high methionine combined with high B vitamins at 3× control levels (M+B+) .

After 10 weeks, consumption of the B vitamin–deficient diets (M+B− and B−) was associated with high degree of hyperhomocysteinemia (Fig. 2) and lowest levels of the three vitamins.30 The most severe hyperhomocysteinemia resulted from the normal methionine, B vitamin–deficient (B−) diet (plasma total homocysteine (243.7 ± 82.0 μM). Combining high methionine and B vitamin deficiency in the M+B− diet attenuated the rise in plasma homocysteine that was achieved by B vitamin deficiency alone. Nevertheless, homocysteine remained significantly elevated with plasma total homocysteine (86 ± 25 μM), in marked contrast to controls with plasma total homocysteine (5.1 ± 1.0 and 4.6 ± 1.4 μM, respectively). The basis for this attenuation is likely due to the allosteric activation of CBS by SAM. Normally, methionine can be conserved during B vitamin deficiency by the drop in SAM that limits CBS, otherwise that would divert methionine from the methylation cycle to the transsulfuration pathway. However, even during B vitamin deficiency, ingested methionine can be converted to SAM, thereby stimulating the clearance of excess homocysteine via transsulfuration.45

Dietary methionine enrichment did not affect plasma methionine concentrations irrespective of B vitamin status (Table 1). Nevertheless, methionine-enriched diets significantly increased aortic lesion area, as shown in Figure 3, which compare hematoxylin–eosin staining of the aortic arch of an ApoE-null mouse fed the

### Table 1. Plasma chemistry after 10 weeks on diet (from Troen et al.30)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>M+B+</th>
<th>M+B−</th>
<th>B−</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folate (ng/mL)</td>
<td>59.4 ± 7.8a</td>
<td>73.0 ± 26.6a</td>
<td>6.3 ± 1.3b</td>
<td>10.0 ± 3.2b</td>
<td>0.001</td>
</tr>
<tr>
<td>Vitamin B12 (pmol/L)</td>
<td>23,877± 6177</td>
<td>46,662± 1126</td>
<td>7122± 1841</td>
<td>7491± 1078</td>
<td>0.001</td>
</tr>
<tr>
<td>PLP (pmol/mL)</td>
<td>160.8 ± 55.3a</td>
<td>191.4 ± 133.9a</td>
<td>33.1 ± 6.8b</td>
<td>16.9 ± 10.5b</td>
<td>0.001</td>
</tr>
<tr>
<td>tHcy (nmol/mL)</td>
<td>4.6 ± 1.4a</td>
<td>5.1 ± 1.0a</td>
<td>86.7 ± 25.3b</td>
<td>243.7 ± 82.0b</td>
<td>0.001</td>
</tr>
<tr>
<td>Methionine (nmol/mL)</td>
<td>13.1 ± 1.6</td>
<td>10.9 ± 1.9</td>
<td>15.4 ± 5.2</td>
<td>11.5 ± 3.3</td>
<td>0.145</td>
</tr>
</tbody>
</table>

Note: Values given are mean ± SD. Values with the same superscript (a, b, or c) are not significantly different; values indicated by different letters (a and b) are significantly different from each other at P < 0.05 by ANOVA with Tukey’s honest squares differences post hoc test. PLP, pyridoxal 5′-phosphate; tHcy, plasma total homocysteine. Copyright (2003) National Academy of Sciences, USA.
Figure 2. The effect of the four dietary regimens on body weight, plasma methionine, plasma tHcy, and aortic plaque area. Fourteen-week-old ApoE-deficient mice fed control diets develop spontaneous lesions as shown by the white bar. The lesion area increased significantly in mice fed methionine-enriched diets (light and dark gray) in comparison with controls. The highest lesion area was attained in mice that were fed the high-methionine, vitamin-deficient M+B− diet, with a mean lesion area that was nearly twice that seen in the control group (dark gray). The mean aortic lesion area in mice fed the vitamin-deficient normal-methionine (B−) diet was not significantly different from the mean basal lesion area in controls (white versus black), despite the fact that the B− group had the highest plasma homocysteine levels. In contrast, the lesion area was significantly higher in mice fed the high-methionine, vitamin-supplemented “M−B−” diet than in controls, despite the fact that homocysteine levels in this group were normal (Table 1). tHcy, plasma total homocysteine. Bars indicated by different letters (a, b, and c) are significantly different within each category; \( P < 0.05 \). Adapted from Troen et al. \(^{30}\) Copyright (2003) National Academy of Sciences, USA.

B vitamin–deficient diet (B−) and the methionine-rich and B vitamin–deficient diet (M+B−).

The most interesting finding in this study was the dissociation of the effects of methionine intake from hyperhomocysteinemia with respect to their relation to the vascular lesions (Fig. 2). The methionine-rich, B vitamin–deficient diet (M+B−) resulted in a nearly twofold increase in lesion area compared with controls where lesion area was 45,923 ± 2804 versus 24 ± 1712 \( \mu \text{m}^2 \), respectively (\( P < 0.05 \)). B vitamin enrichment (M+B+) only partially mitigated this increase despite completely normalizing homocysteine levels: lesion area was 37,936 ± 1298 \( \mu \text{m}^2 \), \( P < 0.05 \) versus controls. It is particularly noteworthy that mice with the most severely elevated homocysteine and normal dietary methionine (B− diet) showed no increase in lesion area compared with controls: lesion area was 23,986 ± 1877 \( \mu \text{m}^2 \). Figure 3 illustrates the histological demonstration of aortic atheromatous plaques in mice fed the M+B+ and B− diets.

A quantitative analysis of the aortic plaque area 10 weeks after initiation of the diets (Fig. 2) showed that both groups receiving methionine-enriched diets have significantly increased area compared to those from a control diet (C) or a diet with B vitamin deficiency (B−) and normal methionine. Curiously, the aortic plaque area was slightly but significantly higher in mice receiving the high methionine vitamin B–deficient diet (M+B−) than those on a diet with excess methionine and excess B vitamin (M+B+) (Fig. 2). This difference could mean that under these conditions, some of the lesions could be prevented by higher than normal vitamin intake.

This interaction between B vitamin and methionine illustrates the critical importance of metabolic context for attributing toxicity to a given metabolite such as methionine or homocysteine. In this study, the harm caused by the identical intake of dietary methionine depended on the availability of B vitamins, presumably by altering fluxes through their interconnected pathways or by compensating...
Figure 3. Atheromatous plaques in the aortic arch of ApoE-deficient mice. Hematoxylin–eosin staining of the aortic arch of an ApoE-null mouse fed the B vitamin–deficient diet (B−, Upper) and the methionine-rich and B vitamin–deficient diet (M+B−, Lower). Although both lesions (P) are classified as fatty streak (initial) lesions, lesions in the M+B− group were significantly larger. L, lumen; M, media; P, plaque. (Scale bar = 100 µm.) From Troen et al.30 Copyright (2003) National Academy of Sciences, USA.

for methionine-induced vitamin depletion. Indeed, the dependence of methionine toxicity on the availability of other nutrients is well documented. For example, adding high dietary methionine to a folate-deficient diet can mitigate the cognitive impairment caused by folate deficiency alone, without affecting homocysteine.46 Similarly, toxic effects of excess dietary methionine have long been known to depend on the balance of other amino acids.47

Although it is difficult to directly translate these findings from animal studies to humans, the principle of metabolic context determining outcomes related to a given level of any single nutrient is broadly applicable. While methionine in the normal range of human intake is not acutely toxic,48 clinical studies in humans clearly show that the effects of methionine loading depend on underlying metabolic conditions.49 As a discussion of clinical trials of B vitamin therapy for homocysteine-lowering and secondary prevention of CVD is beyond the scope of this review, it is noteworthy in this regard that the failure of many randomized clinical trials to prevent secondary CVD by “homocysteine-lowering” through B vitamin supplementation may be due, in part, to a failure to adequately consider the underlying metabolic heterogeneity of hyperhomocysteinemia (i.e., with respect to background B vitamin, protein, and methionine intake, as well as other factors) in designing the studies, thereby attenuating the signal for any benefit in responsive subgroups.50–52 Future research on the long-term influence of high methionine intake on cardiovascular health and other outcomes will need sophisticated approaches to go beyond homocysteine and evaluate overall protein intake, protein quality, and dietary patterns.53 Such efforts will undoubtedly be advanced by employing new metabolomic techniques in evaluating the relationship between methionine intake, metabolism, and disease.54,55

In conclusion, the atherogenic potential of excess dietary methionine that we and others have described in the ApoE-deficient mouse model30,56,57 underscores the central role that methionine and its metabolic products can play in CVD, without any apparent mediation by circulating homocysteine. Although micromolar elevations of circulating homocysteine are frequently described as an “independent” risk factor for CVD in human observational studies, this “independence” is strictly in the epidemiological–mathematical sense of statistical independence from any of the other observed covariates included in a multivariable statistical model, but they do not rule out other plausible explanations for the association. Biologically, fluctuations in circulating homocysteine always follow from changes in flux through the intersecting metabolic pathways that inseparably link methionine to homocysteine. Excessive methionine intake can undoubtedly perturb this metabolism with deleterious vascular consequences. However, whether any specific products of these pathways inflict the vascular damage remains to be determined. Recognition of the potent atherogenic potential of excess methionine intake provides an important point of departure for such investigations. Understanding the metabolic events linking methionine excess to pathology, and their dependence on other underlying metabolic and pathologic conditions, will provide crucial insight into the role these important diet-dependent pathways play in cardiovascular health and disease.
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Conflicts of interest

The authors declare no conflicts of interest.

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