Folic Acid Responsive Postmenopausal Homocysteinemia

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Homocysteinemia is associated with juvenile arteriosclerosis, recurrent thromboembolic complications and osteoporosis. Plasma homocysteine, measured as homocysteine-cysteine mixed disulfide (MDS), has in other than homocysteinemics been reported to be higher in patients with coronary heart or cerebrovascular disease than in controls, and higher in men than in premonopausal women. Here, in groups of normal men and normal premenopausal and postmenopausal women, we measured plasma MDS in the fasting state and four hours after a methionine load (100 mg/kg body weight), before and after four weeks of folic acid therapy at 5 mg daily. In their fasting plasma, postmenopausal women (n = 5) had significantly ($P < 0.05$) higher MDS concentrations than premenopausal women (n = 5) and younger men (n = 5). After the methionine load MDS concentrations in postmenopausal women rose markedly, reaching levels significantly higher than those in younger men ($P < 0.05$), and with no overlap with values in premenopausal women ($P < 0.01$), or in older men (n = 5, $P < 0.01$). Folic acid therapy resulted in substantial reductions (n = 15, $P < 0.01$) of MDS concentrations both before the methionine load (−31%) and after (−28%), though subjects had initially had normal concentrations of serum and erythrocyte folates. We speculate that moderate homocysteinemia might contribute to postmenopausal arteriosclerosis and osteoporosis. Should this prove to be the case, folic acid might be a useful prophylactic.

METHIONINE, an essential amino acid containing sulfur is metabolized to cysteine by means of the transsulfuration pathway. Homocysteine constitutes the juncture between this pathway and the folate, cobalamin, and betaine dependent transmethylation cycle (Fig 1). Deficiencies of certain enzymes involved in these pathways, will result in homocysteinemia, owing to the accumulation, in plasma and tissue, of homocysteine and its two disulfides—homocystine (homocysteine-homocysteine) and homocysteine-cysteine mixed disulfide (MDS).[1]

Homocysteinemia is associated with juvenile arteriosclerosis, recurrent arterial and venous throboembolic manifestations, and osteoporosis. If untreated, more than 50% of affected individuals die before the age of 20 of myocardial infarction, stroke or pulmonary embolism.[2] Experimental homocysteinemia in baboons has shown that homocysteine induces endothelial injury, arteriosclerosis, and thrombosis.[3-5] Homocysteine also interacts with collagen synthesis,[2] an interaction that may be significant in the development of osteoporosis.

Homocysteine concentrations, measured as MDS in plasma from men with ischemic heart disease and controls have been investigated in two studies by Wilcken et al, in one of which,[6] though not in the other,[7] they found significantly higher MDS concentrations in patients than in controls after a methionine load. They suggested that persistent moderate increases in plasma homocysteine might be a risk factor for arteriosclerotic coronary heart disease. In a recent study we measured plasma MDS in patients with carotid artery stenosis, TIAs or strokes, and found significantly higher ($P < 0.05$) concentrations in the patients both before and after a methionine load[8]; female controls (> 50 years) tended to have higher values than males and younger females; three female controls were also given folic acid, 5 mg daily for four weeks, and then reinvestigated, a substantial reduction of the MDS concentrations being found in all three subjects both before and after the methionine load.

Before this study was begun, MDS concentrations in normal women and men had only been reported in young subjects, and to be significantly higher in men than in women.[9,10] We therefore measured plasma MDS in groups of normal premenopausal and postmenopausal women, as well as in age-matched groups of normal men. The subjects were investigated before and after a methionine load and reinvestigated after four weeks of folic acid therapy.

MATERIALS AND METHODS

Five younger men aged 34 to 43 (38.4 ± 1.7, mean ± SEM), five older men aged 45 to 63 (54.0 ± 3.5), five premenopausal women aged 31 to 41 (37.2 ± 1.9) and five postmenopausal women aged 49 to 60 (53.8 ± 1.8) were studied, informed consent was obtained from each subject. All were healthy and receiving no medication, vitamins, or oral contraceptives. Erythrocyte folates, serum folates, and serum cobalamins were within the normal range in all subjects, but younger men had significantly higher concentrations of erythrocyte folates (368 ± 36 nmol/L, mean ± SEM, $P < 0.05$) than did premenopausal women 260 ± 22 nmol/L).

After an overnight fast, a heparinized venous blood sample was drawn at about 8 AM L-methionine (100mg/kg body weight) was then given orally in 200 mL of orange juice. The fast continued and four hours later a second blood sample was drawn. The samples were immediately centrifuged at 4 °C and the plasma stored at - 70° C until analyzed. The methionine load, calculated as L-methionine g/m^2 body surface area, varied individually, being on average higher

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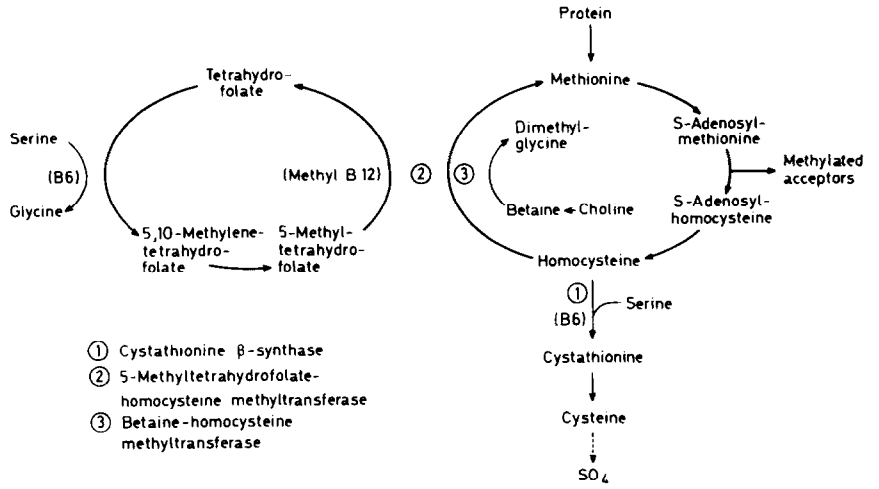

Fig 1. The transsulfuration pathway converts the sulfur atom of methionine into the sulfur atom of cysteine. The transmethylation cycle whereby the methyl group of methionine is transferred to methylated acceptors, and by which methionine is reformed by methylation of homocysteine from 5-methyltetrahydrofolate or betaine.

in younger men (3.94 ± 0.1 g/m^2, $P < 0.05$) and in older men (4.02 ± 0.1 g/m^2, $P < 0.05$) than in premenopausal women (3.54 ± 0.12 g/m^2). The methionine loads given to postmenopausal women (3.68 ± 0.2 m/m^2) did not differ significantly from those given to other groups.

Fifteen of the 20 subjects (four younger men, two older men, four premenopausal women, and five postmenopausal women) were reinvestigated after having received folic acid (KabiVitrum, Stockholm), 5 mg daily for four weeks. Fasting blood samples were also collected from all postmenopausal women eight weeks after discontinuation of folic acid and again two weeks later after another period of folic acid at 5 mg daily.

The plasma amino acid concentrations of methionine, homocysteine-cysteine mixed disulfide, (MDS), cystine, serine, and glycine were measured using a JEOL amino acid analyzer (Modell JLC-6AH) after the samples had been thawed and deproteinized with sulfosalicylic acid, norleucine being used as an internal standard. Statistical significance was assessed using the Mann Whitney rank sum test for unpaired data, and the Wilcoxon rank test for paired data. Linear regression analysis was used for correlations between different variables and r-values were tested with the Student's t-test. A P-value of less than 0.05 was considered significant.

RESULTS

Table 1 shows the plasma amino acid concentrations of methionine, homocysteine-cysteine mixed disulfide (MDS), cystine, serine, and glycine, measured after an overnight fast, and four hours after a 100 mg/kg methionine load both before and after 4 weeks of 5 mg folic acid daily. After an overnight fast and before folic acid therapy, older men had higher concentrations of

Table 1. Plasma Amino Acids (μmol/L, ±SEM) in the Fasting State, and Four Hours After a Methionine Load (100 mg/kg) Before and After Four Weeks of Folio Acid (5 mg Daily) in Normal Men and Women

	Methionine		Homocysteine-cysteine Mixed Disulfide		Cystine		Serine		Glycine	
	Fasting	Post-load	Fasting	Post-load	Fasting	Post-load	Fasting	Post-load	Fasting	Post-load
Younger men n = 5	25 ± 2.8	685 ± 44	3.0 ± 0.2	12.1 ± 1.3	52 ± 2.2	55 ± 3.4	107 ± 6.0	103 ± 5.7	254 ± 18	226 ± 17
Older men n = 5	32 ± 2.9	639 ± 40 †	3.6 ± 0.2	8.5 ± 0.7 †	61 ± 2.7	65 ± 2.4	122 ± 6.0	111 ± 3.2	241 ± 13	201 ± 9
Premenopausal women n = 5	20 ± 1.8	500 ± 35	2.5 ± 0.2	9.2 ± 1.2	46 ± 4.2	56 ± 2.4	121 ± 5.7	108 ± 4.0	266 ± 27	211 ± 15
Postmenopausal women n = 5	24 ± 2.6	738 ± 99	4.4 ± 0.4	16.7 ± 1.0	60 ± 6.0	63 ± 3.8	128 ± 6.8	114 ± 7.8	277 ± 29	213 ± 15
Before folic acid n = 15	24 ± 1.4	658 ± 45	3.5 ± 0.3 †	12.8 ± 1.0 †	55 ± 3.1	60 ± 2.5	119 ± 3.4 •	109 ± 2.9 •	268 ± 13 •	219 ± 8 †
After folic acid n = 15	26 ± 1.4	633 ± 44	2.4 ± 0.2	9.2 ± 0.7	55 ± 2.4	61 ± 2.6	104 ± 6.0	96 ± 5.5	313 ± 20	264 ± 13
Change	+ 4%	− 4%	− 31%	− 28%	± 0%	+ 2%	− 13%	− 12%	+ 17%	+ 21%

*$P < 0.05$
†$P < 0.01$

methionine (32 ± 2.9 μmol/L, mean ± SEM, $P < 0.01$), and MDS (3.6 ± 0.2 μmol/L, $P < 0.05$) than did premenopausal women (20 ± 1.8 and 2.5 ± 0.2 μmol/L resp.). Cystine was higher in older men (61 ± 2.7 μmol/L, $P < 0.05$) than in younger men (52 ± 2.2 μmol/L) and premenopausal women (46 ± 4.2 μmol/L). Postmenopausal women had higher MDS concentrations (4.4 ± 0.4 μmol/L, $P < 0.05$) than younger men (3.0 ± 0.2 μmol/L), and premenopausal women (2.5 ± 0.2 μmol/L). Serine was higher in postmenopausal women (128 ± 6.8, $P < 0.05$) than in younger men (107 ± 6.0 μmol/L).

Four hours after the methionine load, all groups showed significant increases of methionine (>20 fold, $P < 0.01$) and MDS (> 2 fold, $P < 0.01$; Fig 2), a significant reduction of glycine ($P < 0.05$) and a small reduction of serine. Cystine rose significantly only in the premenopausal women ($P < 0.05$). Post load plasma concentrations of methionine were higher in younger men (685 ± 44 μmol/L, $P < 0.01$) and older men (639 ± 40 μmol/L, $P < 0.05$) than in premenopausal women (500 ± 35 μmol/L). Postmenopausal women had the highest mean value (738 ± 103 μmol/L), but due to larger variation the difference was not significant. After the load postmenopausal women had substantially higher MDS concentrations (16.7 ± 1.0 μmol/L) than younger men (12.1 ± 1.3 μmol/L, $P < 0.05$), older men (8.5 ± 0.7 μmol/L, $P < 0.01$) and premenopausal women (9.2 ± 1.2 μmol/L, $P < 0.01$). Older men exhibited higher cystine values (65 ± 2.4 μmol/L, $P < 0.05$) than younger men (55 ± 3.2 μmol/L). The plasma concentrations of serine and glycine did not differ between groups, four hours after the methionine load.

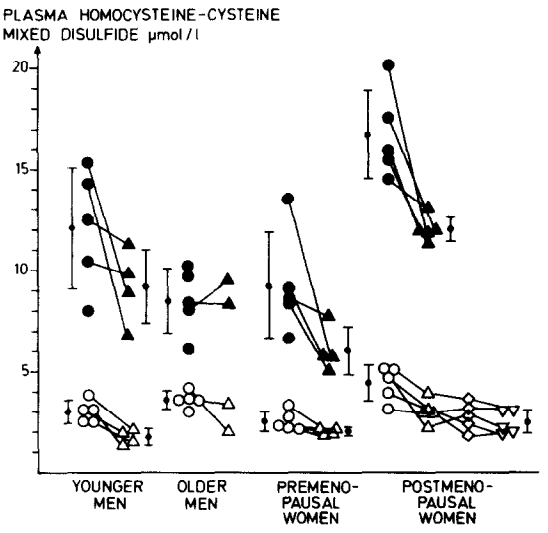

Fig 2. Plasma homocysteine-cysteine mixed disulfide (MDS) in younger and older men, and in premenopausal and postmenopausal women, expressed in μmol/L (individual and mean values ± SD). Key to symbols: open symbols = fasting values; solid symbols = values four hours after a 100 mg/kg peroral load of L-methionine; circles – values before folic acid therapy; triangles – values after four weeks of 5 mg folic acid daily; (postmenopausal women only) rhombs = fasting values eight weeks after termination of folic acid therapy, and inverted triangles = fasting values after a further two weeks of folic acid, 5 mg daily.

In the fasting state there was a correlation between weight and methionine concentrations ($n = 20$, $r = 0.568$, $P < 0.01$). As methionine was given per body weight, the post load correlation was strengthened ($n = 20$, $r = 0.636$, $P < 0.01$). Consequently there was an excellent correlation between methionine dosage, calculated as g/m^2 body surface area and post load plasma methionine concentrations ($n = 20$, $r = 0.724$, $P < 0.001$). No correlation was found, however, between methionine dosages (g/m^2) and post-load MDS concentrations ($n = 20$, $r = -0.122$, not significant). The fasting MDS values were related to the post-load MDS values ($n = 20$, $r = 0.586$, $P < 0.01$).

Four weeks of 5 mg folic acid daily in 15 subjects had varying effects on plasma amino acid concentrations (Table 1). There were no significant changes in methionine and cystine values either before or four hours after the methionine load. MDS concentrations decreased invariably in the 15 subjects, both before the load (3.5 ± 0.3 → 2.4 ± 0.2 μmol/L, $P < 0.01$) and, with one exception, after the load (12.8 ± 1.0 → 9.2 ± 0.7 μmol/L, $P < 0.01$) (Fig 2). Serine decreased significantly—both fasting (119 ± 3.4 → 104 ± 6.0 μmol/L, $P < 0.05$) and post-load (109 ± 2.9 → 96 ± 5.5 μmol/L, $P < 0.05$); but glycine increased—both fasting (268 ± 12.8 → 313 ± 20 μmol/L, $P < 0.05$); and post-load (219 ± 7.5 → 264 ± 12.9 μmol/L, $P < 0.01$). During the treatment, serum folate increased more than five-fold in all subjects (10.1 ± 0.7 → 68.7 ± 3.3 nmol/L, $P < 0.01$).

With the exception of one older man who responded with an increase of the post load MDS value (Fig 2), and a premenopausal woman who had a pronounced increase of serine both pre and post-load, the effect of folic acid was similar in all the subjects. High MDS values, especially after the methionine load, seemed to be reduced most markedly, but even low values were also reduced.

Eight weeks after discontinuation of folic acid, the postmenopausal women still had low fasting MDS concentrations (2.8 ± 0.3 μmol/L) (Fig 2), while their serum folate had decreased from 76 ± 3.5 to 18 ± 2.8 nml/L, a value still above the pretreatment level of 10.8 ± 1.7 nmol/L. After another two weeks of 5 mg folic acid daily, a slight further reduction was noted in

fasting MDS values whose mean reached the same levels as the pretreatment mean value for premenopausal women. The overall reduction was 43% (4.4 ± 0.4 → 2.5 ± 0.3 μmol/L).

DISCUSSION

Since the completion of the present study, Boers and coworkers[11] have published results from a similar investigation, and their findings are partly confirmed by our own. In the fasting plasma as well as after a methionine load (100 mg/kg), premenopausal women in their study had significantly lower MDS concentrations than men, thus confirming earlier reports.[9,10] No differences in MDS values were found between younger and older men. The fasting plasma MDS concentrations of postmenopausal women did not differ from those of men, and were more than three times higher than those found in premenopausal women. After the load, however, the increase in plasma MDS concentrations was considerably greater in postmenopausal women than in any other group, and with no overlap in values with those of premenopausal women. Although the pure disulfide, homocystine, was not detected in fasting plasma from any subject, after the methionine load it was measurable in all postmenopausal women and in most men but, strikingly, in none of the premenopausal women. The homocystine concentrations were significantly higher in postmenopausal women than in both groups of men, and younger men tended to have higher values than older men. On the basis of their findings, Boers et al, speculated, as Wilcken and Gupta[9] had done before them, that the lower plasma homocysteine concentrations, measured as MDS, in premenopausal women than in men of comparable age may constitute protection against vascular disease, and account for its lower prevalence amoung premenopausal women.

The reported differences in MDS concentrations between premenopausal women and younger men are not, however, confirmed by this study, in which the lower MDS values in premenopausal women, both fasting and after the load, were not significantly different from those found in younger men. (Had larger groups been used in this study, these differences might have become significant). As found by Boers and coworkers, fasting MDS concentrations in postmenopausal women were significantly higher than in premenopausal women, but in this study they were also higher than in younger men. We are also able to confirm that postmenopausal women respond to a methionine load with increases of MDS to concentrations significantly higher than those in any other group, and with no overlap in values with those premenopausal women, or (in this study) of older men.

In both studies the average methionine load, even after correction for the body surface area of the dose per kilogram, was similar in the two groups of women, and in the present study, no correlation was found between the methionine loads (g/m^2) and the post load MDS values. Thus, we believe that methionine loads of these magnitudes saturate one or more of the metabolic steps prior to homocysteine in the transsulfuration pathway, and that these results have not been affected by individual differences in the loads. On the other hand, we found a positive and significant relationship between the fasting and the post-load MDS concentrations, indicating that the fasting level also reflects the increase of MDS when the transsulfuration pathway is stressed by a methionine load or a methionine-rich meal.

We are also able to report the original finding that folic acid, administered to nonfolate-deficient normal men and women, almost invariably and significantly reduces the plasma concentrations of homocysteine, measured as MDS, both in the fasting state and after a methionine load. Folic acid has previously only been reported to reduce the high concentrations of homocystein or MDS in homocysteinemic patients,[1,2] renal transplant recipients,[12] and a pair of male twins with coronary heart disease.[7] Our results with folic acid show that, despite normal quantities of serum and erythrocyte folates, the metabolism of methionine, and probably other metabolisms related to the folate system as well, will be affected by excess folic acid. We believe that folic acid, converted in vivo to 5-methyltetrahydrofolic acid simply by a "mass action effect," enhances the remethylation of homocysteine to methionine through the cobalamin-dependent enzyme 5-methyltetrahydrofolate-homocysteine methyltransferase. This hypothesis is supported by the fact that folic acid also significantly decreased serine and increased glycine concentration in plasma. An expected rise of methionine in accordance with this hypothesis was not observed, however.

The postmenopausal increased MDS seems to be secondary to the altered hormonal status, with low estrogens and gestagens, as no corresponding age dependent increase was observed in men. Somewhat at variance with this, however, are reports that, after a methionine load, women on oral contraceptives (OCA) containing estrogen excrete small amounts of homocysteine in urine,[13] and also manifest a significantly increased number of detached endothelial cells in peripheral venous blood, indicating endothelial injury probably due to transient homocysteinemia.[14] It will be interesting if these findings prove to be reproducible, since OCA is known to cause small but significant increases in the incidence of venous thrombosis, myo-

cardial infarction, and stroke[15]—manifestations typical for homocysteinemia.[1,2] It is thus clear that further investigation is needed to determine the role of sex hormones in methionine metabolism.

It is well known, that the frequency of complications from arteriosclerotic vascular disease (eg, myocardial infarction) is much lower in premenopausal women than in men of comparable age, but that after the menopause the frequency increases more rapidly in women than in men.[16,17] Thus any independent risk factor for vascular disease, that might account for these differences between the sexes, is liable to be rare in premenopausal women, common in men, and most common of all in postmenopausal women. The combined data from this study, and those of both Wilcken and Gupta[9] and Boers and coworkers,[10,11] suggest that the plasma concentrations of homocysteine, measured as its disulfides, vary, both between the sexes and within the female sex, in a way that fulfills these criteria. As homocysteine is known to cause endothelial injury[3-5] and arteriosclerosis,[1-5] it is suggestive to speculate that homocysteine or its derivatives, even in concentrations much lower than those found in homocysteinemics, might be worth considering as a risk factor for vascular disease.

Another feature of homocysteinemia is osteoporosis, otherwise most prevalent in postmenopausal women. Homocysteine has been shown to interfere with collagen crosslinking,[1,2] that might lead in turn to a defective bone matrix and osteoporosis. As increased homocysteine concentrations were demonstrated in postmenopausal women, one can also speculate that this might contribute to the hitherto unexplained postmenopausal osteoporosis.

Finally, however, it should be stressed that the fasting MDS concentrations found in postmenopausal women are six to ten times lower than those found in classical homocysteinemia, and that it remains to be established that persistent plasma concentrations of this magnitude are of any pathogenetic importance. If this were the case, however, then folic acid might prove to be an effective prophylactic.

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