Investigation of Relationship Between Reduced, Oxidized, and Protein-Bound Homocysteine and Vascular Endothelial Function in Healthy Human Subjects

John C. Chambers, Per M. Ueland, Melissa Wright, Caroline J. Doré, Helga Refsum, Jaspal S. Kooner

Abstract—Previous studies investigating homocysteine and vascular disease have relied on total plasma homocysteine as the sole index of homocysteine status. We examined the dynamic relationship between vascular endothelial function and concentrations of total, protein-bound oxidized, free oxidized, and reduced homocysteine to identify the homocysteine form associated with endothelial dysfunction in humans. We investigated 14 healthy volunteers (10 men, 4 women). Brachial artery flow–mediated dilatation was measured at baseline and at 30, 60, 120, 240, and 360 minutes after oral (1) L-methionine (50 mg/kg), (2) L-homocysteine (5 mg/kg), and (3) placebo. Plasma concentrations of total, protein-bound oxidized, free oxidized, and reduced homocysteine were measured at each time point, and nitroglycerin-induced dilatation at was assessed at 0, 120, and 360 minutes. Flow-mediated dilatation fell, and concentrations of total, protein-bound oxidized, free oxidized, and reduced homocysteine increased after oral homocysteine and oral methionine (all $P<0.05$ for difference in time course compared with placebo). Flow-mediated dilatation showed a reciprocal relationship with reduced homocysteine during both homocysteine and methionine loading. In both loading studies, peak reduction in flow-mediated dilatation coincided with maximal reduced homocysteine concentrations. In contrast, there was no consistent relationship between flow-mediated dilatation and free oxidized homocysteine, protein-bound oxidized homocysteine, or related species. Nitroglycerin-induced dilatation was unchanged by oral homocysteine and oral methionine ($P>0.10$ compared with placebo). Reduced homocysteine is closely associated with endothelial dysfunction during oral methionine and oral homocysteine loading. Our observations support the hypothesis that reduced homocysteine is the deleterious form of homocysteine for vascular function in vivo and suggest a less important role for other homocysteine species. **(*Circ Res*. 2001;89:187-192.)**

Key Words: homocysteine ■ endothelium ■ atherosclerosis ■ redox ■ nitric oxide

Hyperhomocysteinemia is a major and independent risk factor for vascular disease.[1–5] Homocysteine concentrations exceeding the upper limit of normal (15 μmol/L) are identified in almost 30% of patients with coronary heart disease and are associated with a 3-fold increased risk of subsequent coronary heart disease mortality.[3] In North American and European white populations, 10% of coronary disease may be attributable to elevated homocysteine.[6]

Increasing evidence suggests that the adverse vascular effects of homocysteine are mediated through an action on the endothelium. In healthy human subjects, elevated homocysteine concentrations are associated with impaired endothelium-dependent dilatation, an early manifestation of atherosclerosis.[7–10] Conversely, lowering plasma homocysteine concentrations through treatment with B vitamins is associated with improved vascular endothelial function.[11–13] However, the mechanisms linking homocysteine to endothelial dysfunction are not known.

In plasma, homocysteine exists in reduced, free oxidized, and protein-bound oxidized forms.[14] Most previous studies have relied on total plasma homocysteine as the sole index of homocysteine status, and the active form of homocysteine remains to be determined. Recent observations have indicated that during acute hyperhomocysteinemia, maximal endothelial dysfunction precedes peak total plasma homocysteine concentration[8,9]; these observations imply that short-lived species may mediate the biological effects of homocysteine. Further support for this view comes from in vitro studies, which suggest that reduced, but not oxidized, homocysteine promotes endothelial injury[15] and reacts with NO to form nitrosohomocysteine,[16] which (because of the stability of this nitrosothiol) may decrease the bioactivity of NO.

In the present study, we tested the hypothesis that the reduced form, but not the free oxidized or protein-bound oxidized form, of homocysteine is associated with vascular endothelial dysfunction during hyperhomocysteinemia in humans.

Original received January 2, 2001; revision received May 22, 2001; accepted May 23, 2001.

From the National Heart and Lung Institute (J.C.C., J.S.K.) and the Department of Medical Statistics and Evaluation (M.W.), Imperial College School of Medicine, Hammersmith Hospital, London, UK; the Department of Pharmacology (P.M.U., H.R.), University of Bergen, Armauer Hansen Hus, Bergen, Norway; and the MRC Clinical Trials Unit (C.J.D.), London, UK.

Correspondence to Dr J.S. Kooner, MD, FRCP, Consultant Cardiologist, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK. E-mail j.kooner@ic.ac.uk

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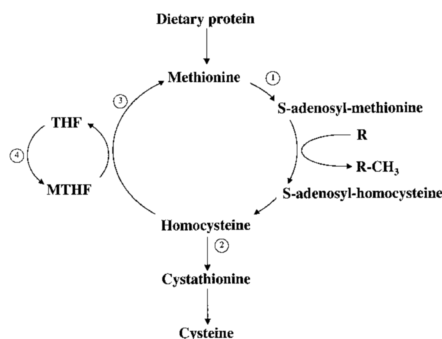

Figure 1. Outline of metabolic pathway for methionine and homocysteine. Numbers indicate the following: 1, *trans*-methylation, the conversion of methionine to homocysteine, with transfer of methyl group to other species (R); 2, *trans*-sulfuration, the irreversible conversion of homocysteine to cysteine via rate-limiting enzyme cystathionine-β-synthase; 3, remethylation, the regeneration of methionine from homocysteine catabolized by methionine synthase, with 5,10-methylenetetrahydrofolate (MTHF, a form of folic acid) and vitamin B_{12} as essential cofactors; 4, regeneration of MTHF from tetrahydrofolate (THF), catabolized by enzyme MTHF reductase.

Materials and Methods

Subjects

We investigated 14 healthy volunteers (10 men, 4 women; mean age 29 [range 19 to 37] years). All subjects were normotensive, with normal serum cholesterol and with no previous history of diabetes or vascular disease. Five of the 14 subjects were cigarette smokers. All subjects abstained from smoking on the day of the study. None were taking medications. The study was approved by the local ethics committee, and all subjects gave informed written consent to participate.

Methods

For each subject, we measured brachial artery vascular responses at baseline and at 30, 60, 120, 240, and 360 minutes after oral (1) L-methionine (50 mg/kg), (2) L-homocysteine (5 mg/kg), and (3) placebo (fruit juice only). Flow-mediated (endothelium-dependent) dilatation was measured at each time point, but nitroglycerin (NTG)-induced (endothelium-independent) dilatation was measured only at 0, 120, and 360 minutes to allow sufficient time for recovery before the next set of vascular measurements. Oral homocysteine and oral methionine (the metabolic precursor of homocysteine, Figure 1) were given as separate metabolic challenges at least 14 days apart. Both oral homocysteine and oral methionine were expected to increase homocysteine concentrations. However, in contrast to the almost immediate rise in plasma homocysteine levels expected after oral homocysteine,[17] oral methionine increases homocysteine concentrations over 4 to 8 hours.[18] We examined the differing time courses and magnitudes of change in homocysteine species after these two challenges to identify the homocysteine form associated with endothelial dysfunction in humans.

Plasma concentrations of reduced homocysteine, free oxidized homocysteine, protein-bound oxidized homocysteine, total homocysteine, methionine, cystathionine, and cysteine were measured at each time point. Additional samples for fasting glucose, total cholesterol, HDL cholesterol, triglycerides, red blood cell folate, and serum vitamin B_{12} were collected for each subject at baseline. Vascular studies were performed on separate days and in random order.

Methionine and Homocysteine Administration

L-Homocysteine was administered in its reduced form. Reduced L-homocysteine was generated by incubation of L-homocysteine thiolactone (Sigma Chemical Co) with 5N NaOH for 5 minutes at room temperature.[17,19] The resultant solution was then neutralized by the addition of HCl, diluted to 200 mL in fruit juice, and administered orally to the subject within 15 minutes. Oral methionine was administered as L-methionine (Sigma) in diluted fruit juice.

Brachial Artery Diameter

Brachial artery vascular responses were measured using a 7.0-MHz linear array transducer, an Acuson 128XP/10 system, and a high-resolution ultrasonic vessel wall tracking system (Vadirec, Ingenious Systems) as previously described.[7,8] In brief, the brachial artery was scanned longitudinally, and a clear section of the vessel was identified. The M-mode cursor was then placed over this point at right angles to the vessel wall. A 5-second segment of the A-mode signal was then routed to the wall tracking system designed to track vessel wall movement on a beat-to-beat basis. Arterial diameter was calculated from the distance between opposite lumen-arterial interfaces, as identified by manual selection of the maximal change in recorded radiofrequency amplitude. After the baseline resting scan, a pneumatic cuff placed at the level of the mid forearm was inflated to 300 mm Hg for 4.5 minutes. Flow-mediated dilatation was determined from a second scan, performed 55 to 65 seconds after cuff deflation. Fifteen minutes was allowed for vessel recovery, after which time a further baseline scan was performed. NTG (400 μg) was then administered, and 4 minutes later, a fourth scan of the brachial artery was undertaken to provide NTG-induced dilatation. Vascular data were stored digitally to enable offline measurement of vessel diameter by two independent observers unaware of the subject's clinical details and the type and stage of the study. The technique for measurement of brachial artery flow–mediated dilatation is reproducible in our laboratory. The intraindividual between-day coefficient of variation for flow-mediated dilatation is 3%, which compares favorably with that in other centers.[20] Flow-mediated dilatation of conduit arteries is endothelium dependent and largely mediated by NO.[21]

Biochemical Measurements

Plasma samples for homocysteine, methionine, cystathionine, and cysteine were collected with each measurement of flow-mediated dilatation.[17,18,22] Blood was collected into two heparinized tubes. One sample was immediately centrifuged at 10 000*g* for 1 minute, deproteinized with sulfosalicylic acid, and then recentrifuged. The supernatant was used for measurement of reduced homocysteine, free homocysteine (the sum of reduced and free oxidized homocysteine), cysteine, cystathionine, and methionine concentrations. The second, untreated plasma sample was used for measurement of total homocysteine and cysteine. Plasma concentrations of total homocysteine, free homocysteine, methionine, and cystathionine were determined by high-pressure liquid chromatography.[17,18,22–24] Reduced homocysteine was determined by mass spectroscopy with deuterated homocysteine as an internal standard by using methodology validated as described by Nexo et al.[25] Concentrations of protein-bound

TABLE 1. Baseline Clinical and Biochemical Measurements of Subjects

	Mean±SD
Age, y	29±6
Body mass index, kg/m^2	26±4
Systolic BP, mm Hg	115±11
Diastolic BP, mm Hg	64±8
Fasting glucose, mmol/L	4.8±0.5
Total cholesterol, mmol/L	4.4±1.0
HDL cholesterol, mmol/L	1.1±0.4
Fasting triglycerides, mmol/L	1.3±0.6
Serum folate, nmol/L	224±44
Vitamin B_{12}, ng/L	394±97

BP indicates blood pressure.

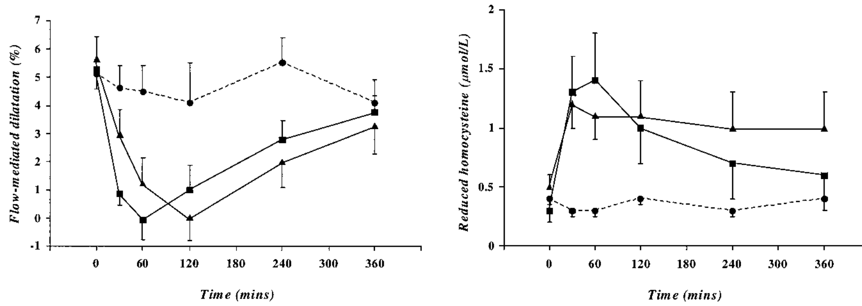

Figure 2. Flow-mediated dilatation and concentrations of reduced plasma homocysteine (mean±SEM) during oral homocysteine (■), oral methionine (▲), and oral placebo (●) loading. Flow-mediated dilatation was reduced, and concentrations of reduced homocysteine were increased during both homocysteine and methionine loading (all $P<0.05$ for difference in time course compared with placebo).

oxidized homocysteine were calculated by subtracting free homocysteine from total homocysteine. Levels of free oxidized homocysteine were calculated by subtracting reduced homocysteine from free homocysteine. For each subject, samples were analyzed in one batch. The within-assay coefficients of variation for measurement of total, free, and reduced homocysteine were 2%, 2%, and 11%, respectively.

Lipid profiles were determined by using an Olympus AU800 multichannel analyzer; vitamin B_{12} and red blood cell folate by were determined by MEIA (Abbott IMX system).

Data Processing and Statistical Analysis

Data were analyzed by using Genstat release 4 and STATA release 6 statistical packages. Continuous data are expressed as mean±SD. Split-unit ANOVA with treatment (oral homocysteine, oral methionine, and oral placebo) and time (0, 30, 60, 120, 240, and 360 minutes) used as factors examined the effects of the administration of oral homocysteine and oral methionine on flow-mediated dilatation, NTG-induced dilatation, and concentrations of plasma thiols. The effect of treatment was assessed relative to the variation between study days within subjects. The effect of time and the interaction between treatment and time were assessed relative to the variation within study days. Pairwise comparison of the three treatments was then performed by using modified *t* tests and the appropriate pooled estimate of the SD from ANOVA. The *P* values obtained for the three multiple comparisons were Bonferroni-adjusted by multiplying each unadjusted *P* value by a factor of 3. The *P* values presented are from the interaction between treatment and time and are intended to investigate whether the time course of the response differs between treatments. Logarithmic transformation was necessary for reduced, free oxidized, protein-bound oxidized, and total homocysteine and for methionine, because these had positively skewed distributions. In addition, Wilcoxon matched-pairs signed rank tests were performed to compare the time of peak reduction in flow-mediated dilatation with the time for the maximum change in concentration of each biochemical species with the use of summary measures for each subject on each study day. Univariate regression analysis was conducted to investigate the relationship between flow-mediated dilatation and concentrations of plasma thiols and methionine. Because repeated measures were made on each participant, robust "sandwich" estimates were made of the between-subject standard errors in the regression analyses. This technique adjusts for any within-subject correlations.[26] Statistical significance was inferred at a value of $P<0.05$.

TABLE 2. Univariate Relationships Between Flow-Mediated Dilatation and Biochemical Indices

	Regression Coefficient	SEM	*P*
Reduced homocysteine*	−2.02	0.42	<0.001
Free oxidized homocysteine*	−1.62	0.33	<0.001
Protein-bound homocysteine*	−1.51	0.48	0.007
Total homocysteine*	−1.87	0.41	0.001
Methionine*	−0.75	0.19	0.002
Cystathionine*	−1.18	0.39	0.01
Total cysteine	0.040	0.012	0.007
Free cysteine	0.002	0.014	0.87
Protein-bound cysteine	0.054	0.013	0.001

Values are expressed as regression coefficient (absolute change in flow-mediated mean±SE per unit change in predictor variable).

*Variable was $\log_e$-transformed for analysis.

Results

The baseline clinical and biochemical measurements of participants are summarized in Table 1. All subjects had normal blood pressure, fasting blood glucose, lipid profile, serum folate, and vitamin B_{12} levels.

Vascular Responses After Oral Homocysteine and Oral Methionine

Flow-mediated dilatation was reduced during both homocysteine and methionine loading ($P=0.009$ and $P=0.03$, respectively, for difference in time course compared with placebo). Impaired flow-mediated dilatation was evident by 30 minutes, was maximal between 60 and 120 minutes, and showed partial

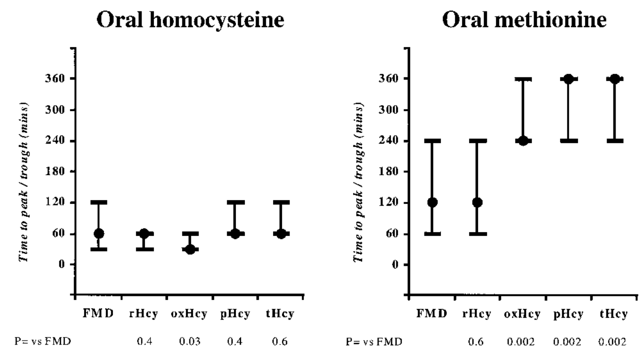

Figure 3. Median time to peak change in flow-mediated dilatation (FMD) and concentrations of total homocysteine (tHcy), protein-bound oxidized homocysteine (pHcy), free oxidized homocysteine (oxHcy), and reduced homocysteine (rHcy) during oral homocysteine and oral methionine loading. *P* values are for comparison between time to peak reduction in flow-mediated dilatation and the time to maximal change in biochemical variable (Wilcoxon matched-pairs signed-rank test). Error bars represent interquartile range. Peak reduction in flow-mediated dilatation coincided with maximal concentrations of reduced homocysteine during both oral homocysteine and oral methionine loading. In contrast, maximal reduction in flow-mediated dilatation coincided with peak concentrations of tHcy, pHcy, and oxHcy during oral homocysteine but not during oral methionine loading.

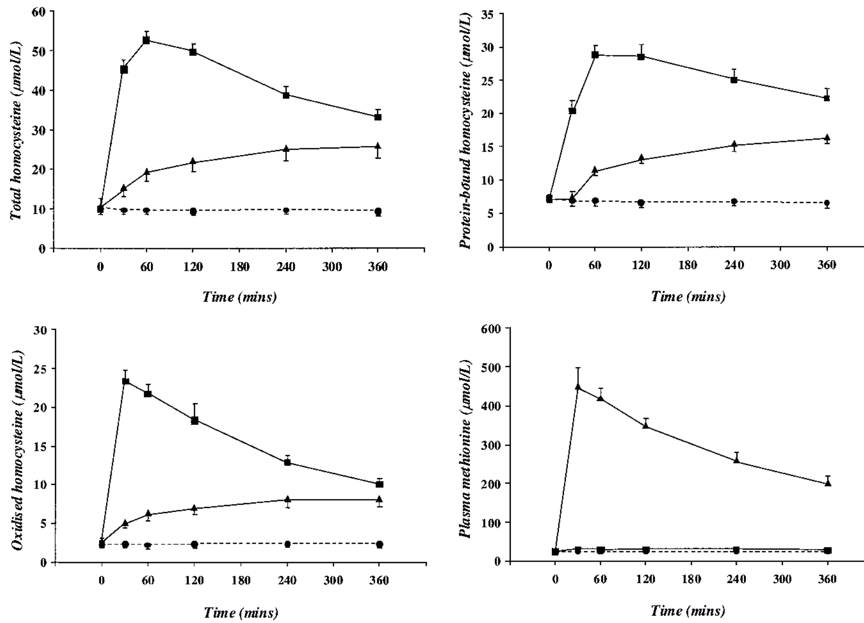

Figure 4. Plasma concentrations of total, protein-bound oxidized, and free oxidized homocysteine and methionine (mean±SEM) during oral homocysteine (■), oral methionine (▲), and oral placebo (●) loading. Mean levels of total, protein-bound oxidized, and free oxidized homocysteine increased during both oral homocysteine and oral methionine loading (all $P<0.001$ for difference in time course compared with placebo). In contrast, methionine concentrations increased after oral methionine ($P<0.001$) but not oral homocysteine ($P=1.00$) compared with placebo.

recovery by 360 minutes after both oral homocysteine and oral methionine (Figure 2). The mean reduction in flow-mediated dilatation was similar during homocysteine and methionine loading, with no evidence of a different time course ($P=0.54$).

In contrast, NTG-induced dilatation was not significantly changed after either oral homocysteine (0 minutes, 19.7+4.4%; 120 minutes, 17.6+5.4%; and 360 minutes, 18.9+3.5%) or oral methionine (0 minutes, 19.9+4.6%; 120 minutes, 17.8+4.8%; and 360 minutes, 19.4+5.5%) compared with measurements after placebo (0 minutes, 18.8+3.7%; 120 minutes, 18.2+4.7%; and 360 minutes, 19.5+4.5%) (both $P=1.0$). Baseline brachial artery diameter and brachial artery flow characteristics were also not significantly influenced by oral homocysteine or oral methionine.

Concentrations of Reduced Homocysteine After Oral Homocysteine and Oral Methionine

Reduced homocysteine concentrations increased during both homocysteine and methionine loading compared with placebo loading (both $P<0.001$ for difference in time course compared with placebo). The increase in reduced homocysteine was evident by 30 minutes, was maximal between 60 and 120 minutes, and showed partial recovery by 360 minutes after both oral homocysteine and oral methionine (Figure 2). The mean rise in reduced homocysteine concentrations was similar during homocysteine and methionine loading, although the time course of the responses differed between the two challenges ($P<0.001$). In regression analysis, there was a close inverse relationship between flow-mediated dilatation and concentrations of reduced

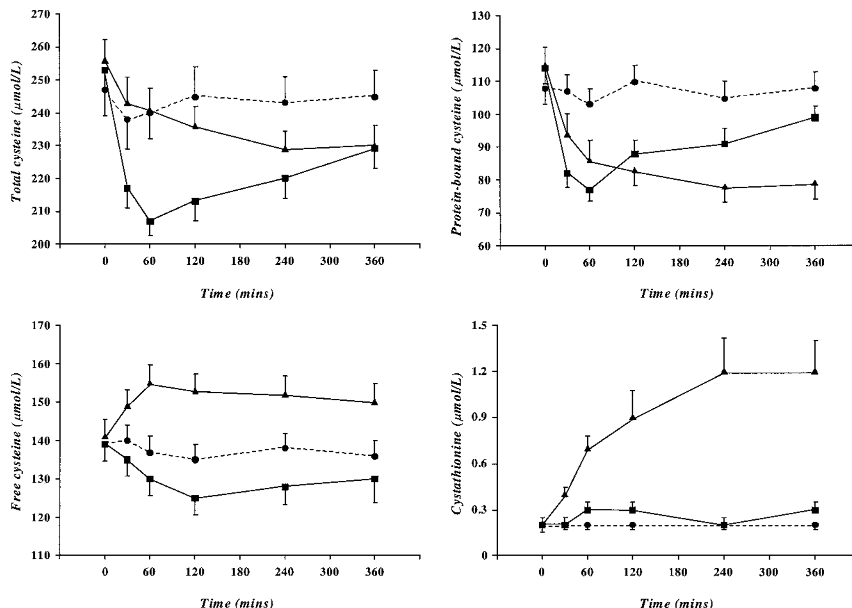

Figure 5. Plasma concentrations of total, protein-bound oxidized, and free cysteine and of cystathionine (mean±SEM) during oral homocysteine (■), oral methionine (▲), and oral placebo (●) loading. Mean levels of total and protein-bound oxidized cysteine fell, and mean levels of cystathionine increased during both oral homocysteine and oral methionine loading (all $P<0.001$ compared with placebo). In contrast, free cysteine concentrations fell after oral homocysteine but increased after oral methionine (both $P<0.001$ compared with placebo).

homocysteine (Table 2). Maximal reduction in flow-mediated dilatation coincided with peak concentrations of reduced homocysteine during both oral homocysteine loading and oral methionine loading (Figures 2 and 3).

Concentrations of Other Homocysteine Species

Mean levels of plasma total homocysteine, protein-bound oxidized homocysteine, and free oxidized homocysteine increased during homocysteine and methionine loading (all $P<0.001$ for difference in time course compared with placebo, Figure 4). However, whereas oral homocysteine and oral methionine had similar effects on flow-mediated dilatation, the increments in concentrations of total, protein-bound oxidized, and free oxidized homocysteine were all greater during oral homocysteine compared with oral methionine loading and followed different time courses ($P<0.001$).

In regression analysis, there was an inverse relationship between flow-mediated dilatation and concentrations of total, protein-bound oxidized, and free oxidized homocysteine (Table 2). However, whereas the peak levels of homocysteine species occurred at a time similar to that for the maximal reduction in flow-mediated dilatation during oral homocysteine loading, concentrations of total, protein-bound oxidized, and free oxidized homocysteine were delayed and still rising at 4 hours after oral methionine, a time at which flow-mediated dilatation was improving (Figures 3 and 4).

Concentrations of Related Metabolites

Although oral homocysteine and oral methionine had similar effects on flow-mediated dilatation, these challenges had very different effects on plasma concentrations of methionine, cystathionine, and cysteine (Figures 4 and 5). Plasma methionine was unchanged after oral homocysteine ($P=1.00$ for difference in time course compared with placebo) but increased by 20-fold after oral methionine ($P<0.001$ compared with placebo). Concentrations of plasma-free cysteine fell after oral homocysteine but increased after oral methionine. Furthermore, there was no consistent relationship between the time to maximal reduction in flow-mediated dilatation and the time of peak change in methionine, cystathionine, or cysteine concentrations. In regression analysis, methionine and cystathionine were less strongly related, and free cysteine concentrations were unrelated to flow-mediated dilatation (Table 2).

Discussion

The results of the present study show that the reduced form of homocysteine is closely associated with vascular endothelial dysfunction induced by both oral methionine and homocysteine loading. Our observations support the hypothesis that reduced homocysteine is the deleterious form of homocysteine for vascular function in vivo and argue against a significant vascular effect for other homocysteine species.

Investigations of the possible mechanisms underlying the association between homocysteine and vascular disease have been limited by the fact that little is known about which homocysteine species exerts vascular effects in vivo. Most previous studies investigating the link between elevated homocysteine concentrations and vascular disease have been based on measurements of total plasma homocysteine,[1–4,7–12] because routine laboratory assays do not distinguish between the protein-bound oxidized, free oxidized, and reduced forms of homocysteine.[14,22,23] In the present study, we observed an inverse and dynamic relationship between the reduced form of plasma homocysteine and endothelial function. Oral methionine and oral homocysteine induced a rise in reduced homocysteine concentrations and a fall in brachial artery endothelium-dependent dilatation. In both time-course studies, peak concentrations of reduced homocysteine coincided with the maximal reduction in flow-mediated dilatation, and regression analysis showed an inverse relationship between reduced homocysteine concentrations and flow-mediated dilatation. These observations suggest that vascular endothelial dysfunction during hyperhomocysteinemia may be accounted for by the reduced form of homocysteine. Additional independent support for this view comes from physiological studies showing that the effects of homocysteine on vascular endothelial function are mediated by oxidation stress mechanisms[7,10] and from in vitro studies demonstrating that it is the reduced, but not the oxidized, form of homocysteine that promotes the generation of oxygen-derived free radicals and reacts with NO.[15,16,27]

Although a separate effect of free oxidized or protein-bound oxidized homocysteine on endothelial function cannot be excluded, this possibility is unlikely because the relationship between these homocysteine species and flow-mediated dilatation was not consistent between the studies. After oral homocysteine, peak concentrations of total, protein-bound oxidized, and free oxidized homocysteine occurred within 60 minutes, a time similar to that for the maximal reduction in flow-mediated dilatation. In contrast, peak concentrations of total, protein-bound oxidized, and free oxidized homocysteine were delayed and were still rising at 4 hours after oral methionine, a time at which flow-mediated dilatation was improving. A threshold or other nonlinear relationship between homocysteine forms and flow-mediated dilatation is unlikely to account for these findings because previous studies show that the relationship between homocysteine and flow-mediated dilatation is graded and dose dependent.[8] A separate effect of cysteine, which has been linked to coronary heart disease in some epidemiological studies,[28] is also unlikely because free cysteine concentrations fell during oral homocysteine loading but increased during oral methionine loading. Furthermore, because homocysteine loading was not associated with a significant rise in plasma methionine concentrations, our results exclude a role for methionine or methionine-dependent methylation in the observed vascular responses.

In the present study, there was no constant relationship between total plasma homocysteine concentrations and vascular endothelial function. In particular, we found that during hyperhomocysteinemia induced by oral methionine, maximal reduction in flow-mediated dilatation occurred at 2 hours and then showed improvement by 6 hours, even though concentrations of total homocysteine were still rising. These findings indicate that total plasma homocysteine concentrations may not be a consistent marker of the biological activity of homocysteine in vivo. This important limitation may have

obscured the interpretation of the relationship between homocysteine and vascular disease in previous experimental and clinical studies.[29–32] Because the relationship between total and reduced homocysteine concentrations varies between subjects and under differing metabolic conditions,[17,18] total homocysteine may only be a marker of reduced homocysteine and vascular risk under certain circumstances. Our results suggest that reduced homocysteine may prove to be a more valid measurement for future epidemiological and experimental studies. This possibility needs to be examined in specific studies using simultaneous measurements of reduced and total homocysteine.

In summary, we have shown that the reduced form of homocysteine is closely associated with vascular endothelial dysfunction, which is associated with elevated homocysteine in humans. Identification of reduced homocysteine as the deleterious form of homocysteine for vascular function in vivo and the resultant changes in cellular redox potential will provide new insight into the mechanisms linking hyperhomocysteinemia and atherosclerosis.

Acknowledgments

This study was supported by British Heart Foundation project grant PG99204 and European Union Commission Demonstration Project Contract No. BMH4-98-3549. We are indebted to Andrew McGregor and Jeff Jean-Marie for their assistance with the vascular studies.

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