The effects of oral methionine and homocysteine on endothelial function

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Abstract

Background—Raised homocysteine is a risk factor for vascular disease. Homocysteine is formed from methionine, and dietary manipulation of homocysteine in primates and humans with oral methionine is associated with endothelial dysfunction. A cause–effect relation has not been clearly established.

Aim—To study the effect of oral methionine and then oral homocysteine on endothelial function.

Methods—22 healthy adults were recruited for two randomised crossover studies, each containing 11 subjects. Endothelial function was determined by measuring forearm blood flow in response to intra-arterial infusion of acetylcholine (endothelium dependent) and sodium nitroprusside (endothelium independent). Subjects received methionine or placebo (study 1), or homocysteine or placebo (study 2). Methionine and homocysteine were determined at baseline and $t = 4$ hours. Endothelial function was determined at four hours. The responses to the vasoactive substances are expressed as the area under the curve of change in forearm blood flow from baseline.

Results—Study 1: plasma methionine and homocysteine concentrations increased significantly versus placebo. The increases were associated with a reduction of endothelium dependent responses (mean (95% confidence interval), arbitrary units), from 48.8 (95% CI 36.4 to 61.2) to 29.9 (95% CI 18.0 to 41.1), $p < 0.04$; endothelium independent responses were unchanged. Study 2: homocysteine concentration increased significantly while methionine remained unchanged. Endothelium dependent responses were reduced from 34.6 (95% CI 20.6 to 48.6) to 22.8 (95% CI 12.0 to 33.6), $p < 0.03$.

Conclusions—Homocysteine and not methionine is responsible for the changes in endothelial function. This supports the hypothesis that homocysteine promotes atherosclerosis by inducing endothelial dysfunction.

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Moderate elevation of total homocysteine concentration is an established independent risk factor for the development of vascular disease.¹ Homocysteine is formed during methionine metabolism, and oral administration of methionine will increase plasma homocysteine. An exaggerated homocysteine rise following methionine loading may identify individuals at increased risk of vascular disease.² There are various mechanisms by which increased homocysteine may promote vascular disease. One appears to be endothelial damage induced by homocysteine,³ which may be mediated by hydrogen peroxide formation.⁴ Damage to the endothelium is important because endothelial dysfunction is an early and reversible indicator of the atherogenic process.⁵

In primates, dietary supplementation with methionine results in increased homocysteine and in the development of changes consistent with atherosclerosis.⁶ In healthy subjects, increasing homocysteine with oral methionine (100 mg/kg) impairs endothelium dependent vascular responses acutely.^{7,8} It has been assumed that the change in homocysteine is responsible for these effects, but a cause–effect relation has not been fully established. It is possible that the changes reflect an increase in methionine concentration. To clarify this we performed two studies in healthy volunteers,

investigating the effect of oral methionine and then oral homocysteine on methionine concentration, homocysteine concentration, and endothelial function.

Methods

SUBJECTS

Twenty two healthy non-smoking volunteers (19 male and three female), mean age 25 years (range 20–35 years), were recruited for two separate study groups of 11 subjects. The subjects were recruited from staff within our department. Each underwent a medical history, examination, ECG, and routine laboratory tests. The study was approved by the ethics committee of The Queen's University, Belfast. All subjects gave written informed consent.

DESIGN

Endothelial function was assessed by measuring changes in forearm blood flow using venous occlusion plethysmography in response to local infusion of vasoactive substances. Each subject fasted overnight and abstained from alcohol and caffeine containing products for 12 hours before the study.

In study 1, 11 subjects (nine male, two female) were randomised to receive methionine (100 mg/kg) in fruit juice or placebo

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Table 1 Total methionine and homocysteine concentrations, basal blood flow, and area under the curve (AUC) for change in blood flow following oral methionine and homocysteine

		Study 1		Study 2	
		Placebo	Methionine	Placebo	Homocysteine
Total methionine ($\mu\text{mol/l}$)	Baseline		22 (20.5 to 23.5)		23.2 (19.1 to 27.3)
	t=4 hours		545 (495 to 595)*		30.4 (24.5 to 36.3)
Total homocysteine ($\mu\text{mol/l}$)	Baseline	8.4 (6.0 to 10.8)	8.5 (6.1 to 10.8)	8.3 (6.6 to 10.0)	8.8 (7.9 to 9.7)
	t=4 hours	9.2 (7.2 to 11.2)	27.3 (21.0 to 33.6)*	8.2 (6.1 to 10.1)	35.7 (30.5 to 40.9)*
Basal FBF ($\text{ml}/100 \text{ ml}/\text{min}$)		2.9 (2.2 to 3.6)	2.9 (2.1 to 3.7)	4.0 (3.3 to 4.7)	3.9 (3.2 to 4.6)
AUC endothelium independent change in FBF		31.7 (16.3 to 47.1)	33.0 (14.1 to 51.9)	26.7 (20.8 to 32.6)	22.5 (17.4 to 27.6)
AUC endothelium dependent change in FBF		48.8 (36.4 to 61.2)	29.9 (18.0 to 41.1)†	34.6 (20.6 to 48.6)	22.8 (12.0 to 33.6)†
Difference in AUC from placebo (endothelium dependent)			19 (4.0 to 34)		13 (1.2 to 24)

Data are mean (95% CI).

* $p < 0.0001$ v baseline and placebo; † $p < 0.05$ v placebo.

FBF, forearm blood flow.

(methionine-free fruit juice). This study employed a single (operator) blind crossover design with a minimum of two weeks between studies. Because of the distinctive taste of methionine a research assistant not involved in the study performed randomisation in a separate room. The subjects were also asked not to comment on the taste of the preparation to the investigator. In study 2, 11 subjects (10 male, one female) were randomised to receive either L-homocysteine (67 $\mu\text{mol/kg}$) or placebo. This study employed a randomised, double blind crossover design with a minimum of two weeks between studies.

Randomisation was performed at time $t = 0$. On each study day, venous blood samples were taken and placed in ice at baseline ($t = 0$) and after four hours ($t = 4$) for plasma methionine and total homocysteine measurement. Forearm blood flow was assessed at four hours after randomisation. The subjects consumed a standard meal after randomisation and refrained from food until completion of the study protocol. The brachial artery was cannulated 30 minutes before the first recording to allow the area to normalise.

FOREARM BLOOD FLOW MEASUREMENTS

Studies took place in a temperature controlled room (24–26°C). A 27 gauge needle was inserted under local anaesthetic into the non-dominant brachial artery to allow local drug infusion. The subjects were supine, with arms resting on a support slightly above heart level. Forearm blood flow was measured by strain gauge venous occlusion plethysmography. A mercury in Silastic strain gauge was coupled to an electronically calibrated plethysmograph (Medasonics model SPG16, Newark, California, USA). The output was transferred to a Macintosh personal computer (Performa 630, Apple Computer, Newark, California, USA) with a MacLab analogue to digital converter and CHART software (v. 3.4.3) (AD Instruments, Hastings, Sussex, UK). The mean of five consecutive forearm blood flow measurements was taken for statistical evaluation. Forearm blood flow was expressed as $\text{ml}/100 \text{ ml}$ forearm volume/min.

Following 30 minutes of rest during which 0.9% saline was infused, basal forearm blood flow was measured. Sodium nitroprusside was infused intra-arterially in incremental doses (3, 6, 9, and 12 nmol/min), each for three minutes, to assess endothelium independent vasodilata-

tion, with forearm blood flow measured in the last minute of each infusion. After a washout period of 20 minutes, basal forearm blood flow was measured again. Acetylcholine was then infused intra-arterially in incremental doses (60, 120, 180, and 240 nmol/min), each for three minutes, to assess endothelium dependent vasodilatation. Forearm blood flow was measured as above. All infusions were given at a rate of 1 ml/min using a constant rate infusor. The doses used have no systemic effect on heart rate or blood pressure.⁹ Forearm blood flow measured in the non-cannulated arm acted as a control, showing no systemic action of the infused drugs.

PREPARATION OF HOMOCYSTEINE

L-homocysteine thiolactone was purchased from Sigma Chemical Company (Poole, Dorset, UK). It was converted to the reduced form by incubating with 5 mol/l NaOH for five minutes at room temperature. The solution was placed on ice, neutralised with 5 mol/l HCl, diluted to 200 ml (pH 4–5), and given orally.¹⁰

PLASMA HOMOCYSTEINE AND METHIONINE

Plasma was separated within 20 minutes of venepuncture and stored at -70°C until analysis. Plasma homocysteine was measured according to the method of Ubbink and colleagues using high performance liquid chromatography (HPLC) with fluorescence detection.¹¹ At a plasma homocysteine concentration of 7.87 $\mu\text{mol/l}$, the interassay coefficient of variation (CV) was 6.8%, with an intra-assay CV of 2.6%. Plasma methionine concentration was measured by HPLC using a standard technique.

STATISTICAL ANALYSIS

The changes in homocysteine and methionine concentrations were compared using a paired samples t test, as was basal forearm blood flow between study days. The responses to the vasoactive substances are expressed as the area under the curve (AUC) of change in forearm blood flow from baseline. Results were then analysed using a paired sample t test. This method of analysis avoids making multiple comparisons.¹² The mean of the differences from placebo is also shown and the data are expressed in arbitrary units.

Figure 1 Changes in methionine and homocysteine concentrations (μmol/l) at baseline and four hours after oral homocysteine.

Data are expressed as means with 95% confidence intervals (CI). Differences were considered significant at a probability value of $p < 0.05$.

Results

Baseline plasma homocysteine was unchanged on both study days, suggesting no carryover effect. Four hours after oral methionine there was a significant increase in concentrations of both methionine (from 22 μmol/l (95% CI 20.5 to 23.5 μmol/l) at baseline to 545 μmol/l (95% CI 495 to 595 μmol/l) at $t = 4$; $p < 0.0001$) and homocysteine (from 8.5 μmol/l (95% CI 6.1 to 10.8 μmol/l) at baseline to 27.3 μmol/l (95% CI 21.0 to 33.6 μmol/l) at $t = 4$; $p < 0.0001$) (table 1).

Figure 2 Change in endothelium dependent forearm blood flow responses measured four hours after oral methionine and homocysteine. Data are means, error bars = SEM.

Figure 3 Area under the curve for change in endothelium dependent forearm blood flow responses plus the mean of the differences, measured four hours after oral methionine and homocysteine. Data are means (95% CI).

Following oral homocysteine supplementation, plasma homocysteine increased significantly from baseline, from 8.8 μmol/l (95% CI 7.9 to 9.7 μmol/l) to 35.7 μmol/l (95% CI 30.5 to 40.9 μmol/l) at $t = 4$ hours; $p < 0.0001$; however, methionine concentration was not significantly altered (23.2 μmol/l (95% CI 19.1 to 27.3 μmol/l) at baseline *v* 30.4 μmol/l (95% CI 24.5 to 36.3 μmol/l) at $t = 4$ hours; NS) (fig 1, table 1).

There was no change in basal forearm blood flow between experiment days, and the flow was unchanged before each drug infusion. Blood flow in the control arm did not change in response to any drug infusion, thus confirming that the effects were confined to the experimental forearm.

In study 1 there was a significant reduction in endothelium dependent forearm blood flow responses compared with placebo at $t = 4$ hours after oral methionine (from 48.8 units (95% CI 36.4 to 61.2 units) to 29.9 units (95% CI 18.0 to 41.1 units); $p < 0.03$) (fig 2, table 1). Endothelium independent responses were not different from placebo (33.0 units (95% CI 14.1 to 51.9 units) *v* 31.7 units (95% CI 16.3 to 47.1 units)) (table 1).

In study 2 endothelium dependent responses four hours after oral homocysteine were reduced compared with placebo (from 34.6 units (95% CI 20.6 to 48.6 units) to 22.8 units (95% CI 12.0 to 33.6 units); $p < 0.03$) (fig 3, table 1). There was an inverse correlation between the increase in homocysteine from baseline and the reduction in the AUC for change of forearm blood flow. This approached significance ($r = 0.6$, $p = 0.057$). Endothelium

independent responses were not different from placebo (22.5 units (95% CI 17.4 to 27.6 units) *v* 26.7 units (95% CI 20.8 to 32.6 units)) (table 1).

Discussion

Our main findings were that oral methionine increased plasma methionine and homocysteine concentrations with reduced endothelium dependent vasodilatation at $t = 4$ hours. Following oral homocysteine, homocysteine increased significantly, and endothelium dependent responses were impaired; however, methionine concentration did not change significantly. These findings suggest that the increases in homocysteine were responsible for the impairment of endothelium dependent vasodilatation. Previously reported studies have used methionine to increase homocysteine; this is the first report investigating the effect on endothelial function of increasing homocysteine by oral homocysteine administration. Our data suggest that homocysteine is causal.

A moderate increase in homocysteine is a risk factor for the development of vascular disease.¹ Hyperhomocysteinaemia in humans is associated with impaired endothelium dependent vasodilatation before overt evidence of vascular disease.¹³ Impairment of endothelium dependent vasodilatation is important, as endothelial dysfunction is an early and reversible indicator of the atherogenic process.⁵

Methionine, an essential amino acid, is converted to homocysteine by demethylation. Homocysteine then undergoes conversion to cysteine by transsulphuration or is remethylated back to methionine. Several animal studies have investigated dietary modification of homocysteine concentration. In monkeys, diet induced hyperhomocysteinaemia was associated with patchy desquamation of the vascular endothelium³ plus altered vascular function, abnormal platelet function, and decreased thrombomodulin dependent activation of protein C.⁶ In rabbits, changes in vessel walls consistent with the development of atherosclerosis developed following oral methionine.¹⁴

Experimental hyperhomocysteinaemia in humans following oral methionine (100 mg/kg) resulted in acute impairment of endothelium dependent vasodilatation.^{7,8} We have previously shown that a much lower concentration of methionine (250 mg orally) had no effect on homocysteine or on endothelium dependent vasodilatation acutely. Also, in an open study of eight volunteers we found that methionine (100 mg/kg) given for seven days was associated with increased homocysteine, but there was no impairment of endothelium dependent vasodilatation.^{15,16}

It has been assumed that the acute changes in endothelium dependent vasodilatation resulted from the increase in homocysteine, but a cause-effect relation has not been fully established. Using flow mediated dilatation, Bellamy and colleagues showed impaired endothelium dependent vasodilatation following oral methionine.⁸ Serial measurements demonstrated that this impairment was maximal at four hours

after randomisation and appeared to normalise at eight hours. At this time methionine concentrations were falling while homocysteine was continuing to rise. These data could suggest a methionine effect. Kanani and colleagues found impaired endothelium dependent vasodilatation following methionine loading in both conduit and resistance vessels.¹⁷ This was maximal at eight hours after ingestion of methionine, which is more suggestive of homocysteine being causative. It is possible that the changes detected resulted from an increase in methionine itself or an intermediate product in the formation of homocysteine. All of these compounds contain a sulphhydryl group and are capable of similar reactions to homocysteine. Both groups of investigators raised this point in their discussion.

We attempted to clarify this issue by giving homocysteine orally to increase plasma homocysteine. We showed that oral methionine increased plasma methionine concentration 25-fold and homocysteine fourfold at $t = 4$ hours. At this time endothelium dependent vasodilator responses were impaired. Following oral homocysteine there was a similar increase in homocysteine, with only a minor increase in methionine; again endothelium dependent responses were reduced. On theoretical grounds we might have expected a greater increase in methionine following oral homocysteine because of remethylation, but our data did not show this. Our data support the view that raised plasma homocysteine is a cause rather than an effect of impaired endothelium dependent vasodilatation.

There are many possible mechanisms by which the acute change in homocysteine may produce impaired endothelium dependent dilatation. In vitro work suggests that homocysteine damage to endothelial cells is hydrogen peroxide mediated.⁴ Chambers and colleagues and Kanani and associates showed that vitamin C, an aqueous phase antioxidant, prevented the impairment of endothelium dependent vasodilatation following oral methionine.^{17,18} These data support the view that the changes may reflect increased oxidative stress. Another possibility is that methylation of other compounds may occur. L-arginine is the principal substrate for the enzyme nitric oxide synthase; methylation of L-arginine produces asymmetric dimethylarginine (ADMA), a naturally occurring competitive inhibitor of nitric oxide synthase.¹⁹ The formation of ADMA could affect vascular function adversely. In monkeys fed a hyperhomocysteinaemic diet there was an increase in ADMA concentration that correlated with the increase in homocysteine.²⁰ There was also an inverse correlation between the increase in ADMA concentration and a reduction in carotid artery responses to acetylcholine. While the formation of ADMA could theoretically result in reduced endothelium dependent vasodilatation this remains an open question *in vivo*.²¹ In our study we did not try to investigate mechanisms but attempted to clarify the cause-effect relation.

A potential drawback of this study was that a matched placebo was not available for study 1 owing to the distinctive taste of methionine. To try to circumvent this problem, randomisation was performed by an assistant not participating in the study protocol. This took place in a separate room and the subjects were specifically asked not to comment on the taste to the study operator. This design has been used in similar work, and our findings regarding oral methionine and endothelial function support the published data.

CONCLUSIONS

We have shown that in healthy subjects oral methionine increased plasma homocysteine and methionine concentration, with a concurrent reduction in endothelium dependent vasodilatation. Oral homocysteine increased plasma homocysteine, with little effect on methionine, and also led to impaired endothelium dependent vasodilatation. These data suggest that homocysteine is the causative agent and support the theory that homocysteine may produce vascular disease through endothelial damage.

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