Elevation of asymmetrical dimethylarginine may mediate endothelial dysfunction during experimental hyperhomocyst(e)inaemia in humans

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ABSTRACT

Hyperhomocyst(e)inaemia is associated with endothelial dysfunction in animals and humans. Mechanisms responsible for endothelial dysfunction in hyperhomocyst(e)inaemia are poorly understood, but may involve impaired bioavailability of endothelium-derived nitric oxide (NO). We hypothesized that acute elevation of homocyst(e)ine by oral methionine loading may stimulate the formation of asymmetrical dimethylarginine (ADMA), an endogenous inhibitor of NO synthase, due to a transmethylation reaction during the formation of homocyst(e)ine from methionine. We studied nine healthy human subjects (five males, four females) aged 29 ± 2 years. Flow-mediated vasodilation (FMD) in the brachial artery (endothelium-dependent) and vasodilation induced by nitroglycerine (endothelium-independent) were measured with high-resolution ultrasound before and 8 h after oral methionine (100 mg/kg in cranberry juice) or placebo (cranberry juice), on separate days and in random order. Plasma homocyst(e)ine and ADMA concentrations were measured by specific HPLC methods. After a methionine bolus, elevation of homocyst(e)ine (28–4 ± 3–5 l mol/l) was associated with an increased plasma concentration of ADMA (2–03 ± 0–18 l mol/l) and reduced FMD (1–54 ± 0–92 %). Placebo had no effect on these parameters. There was a significant inverse linear relationship between ADMA concentration and FMD (r = −0.49; P < 0.05), which was stronger than the relationship between the homocyst(e)ine concentration and FMD (r = −0.36; not significant). We conclude that acute elevation of the homocyst(e)ine concentration impairs vascular endothelial function by a mechanism in which an elevated concentration of ADMA may be involved. This finding may have importance for understanding the mechanism(s) leading to homocyst(e)ine-associated vascular disease, and its potential treatment.

INTRODUCTION

Hyperhomocyst(e)inaemia, a major and independent risk factor for cardiovascular disease, is associated with endothelial dysfunction [1,2], the mechanisms of which are not fully understood. The term ‘hyperhomocyst(e)inaemia’ is used in this paper to indicate that plasma homocyst(e)ine assays measure the total concentration of

Key words: atherosclerosis, coronary disease, endothelial function, nitric oxide, ultrasound.

Abbreviations: ADMA, asymmetrical dimethylarginine; DDAH, dimethylarginine dimethylaminohydrolase; FMD, flow-mediated vasodilation; SDMA, symmetrical dimethylarginine.

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thiol, disulphide and mixed-disulphide adducts of homocysteine. In high concentrations, homocyst(e)ine may be directly toxic to cultured endothelial cells [3], but differences in homocyst(e)ine concentrations observed in clinical trials between cases and controls were much smaller [4,5]. Evidence suggests that decreased biological activity of endothelium-derived nitric oxide (NO) may be involved in homocyst(e)ine-associated endothelial dysfunction (for a review, see [6]). Stamler et al. [7] postulated that the redox activity of homocyst(e)ine may contribute to enhanced oxidative inactivation of NO.

Based on the results of a study in non-human primates, we have hypothesized that the generation of asymmetrical dimethylarginine (ADMA), an endogenous inhibitor of NO synthase [8], may be a novel mechanism contributing to the endothelial dysfunction associated with hyperhomocyst(e)inaemia [9]. An elevated plasma level of ADMA is a risk factor for endothelial dysfunction in hypercholesterolaemia [10] and atherosclerosis [11]. ADMA is synthesized by methylation of the side-chain guanidino nitrogen atoms of L-arginine residues within proteins, due to the action of methyltransferases that utilize S-adenosylmethionine as a methyl group donor [12]. S-Adenosylmethionine is an intermediate in the demethylation of methionine to homocyst(e)ine, which is the major pathway supplying methyl groups for various methylation reactions (for a review, see [13]).

Acute hyperhomocyst(e)inaemia can be induced by oral methionine loading in healthy humans, and produces endothelial dysfunction [14–16]. Our hypothesis was that increased availability of S-adenosylmethionine during experimental hyperhomocyst(e)inaemia induced by oral methionine loading may stimulate the formation of ADMA, which will then induce endothelial dysfunction by inhibiting endothelial NO synthase. The present study was undertaken to test whether elevation of ADMA plasma levels is associated with endothelial dysfunction during experimental hyperhomocyst(e)inaemia (induced by acute methionine loading) in healthy humans.

METHODS

Subjects and study protocol
This was a two-phase, randomized, double-blind, placebo-controlled, cross-over study comparing the effects of oral L-methionine and placebo on endothelial function, with a washout period of at least 1 week between the two study days. The investigation conformed with the principles outlined in the Declaration of Helsinki. The studies were conducted after obtaining written informed consent from each subject, and with the approval of the University of Iowa Institutional Review Board. Nine healthy subjects, without risk factors for, or clinical evidence of, atherosclerosis, were recruited by advertisement. No subject received vasoactive drugs in the week before the study, and all abstained from alcohol for 24 h, and from caffeine for at least 12 h, before any measurements. Subjects were admitted to the University of Iowa General Clinical Research Center in the evening, and studied in a fasted state the next morning. Studies were performed in a quiet room maintained at a constant temperature of between 22 and 25 °C. An antecubital vein was cannulated with a 22G catheter for blood sampling. Subjects received oral L-methionine (100 mg/kg; Ajinomoto, Teaneck, NJ, U.S.A.) dissolved in cranberry juice, or cranberry juice alone, at approx. 07.00 hours. A standard breakfast containing 58 mg of L-methionine and 5 g of fat was served 2 h after methionine or placebo administration. Conduit vessel vasomotor function was assessed 8 h after L-methionine/placebo administration. Venous blood for assay of plasma homocyst(e)ine and methionine was obtained before and 8 h after L-methionine/placebo administration.

Endothelium-dependent vasodilation
To study endothelial function in conduit vessels, flow-mediated vasodilation (FMD) of the brachial artery was induced using reactive hyperaemia as a stimulus [2,17,18]. A 5 cm length of the brachial artery was imaged in longitudinal section above the antecubital fossa using an ultrasound-Doppler machine with a 7.5 MHz linear array transducer (Toshiba SSA-270, Andover, MA, U.S.A.). In order to improve reproducibility, we used the same ultrasonographer for image acquisition, a standard arm support and identical probe distance from the antecubital fossa (2.5 cm proximal). Arterial flow velocity measurements were made by a pulsed Doppler signal at an ~70° angle to the vessel. Measurements were obtained for 20 s at baseline. An occluding forearm cuff placed 5 cm below the antecubital fossa was inflated to 50 mmHg above systolic pressure for 5 min and then released to induce reactive hyperaemia. Recordings were made 5, 60 and 120 s after the onset of reactive hyperaemia. Brachial artery diameter and flow velocity were also measured before and for 6 min after sublingual administration of nitroglycerine spray (400 μg). Blood pressure was recorded using an automated sphygmomanometer (LifeStat 200; Physio Control, Redmond, WA, U.S.A.).

Biochemical analyses
Plasma homocyst(e)ine assays were performed in the University of Iowa General Clinical Research Center Core Lab, using a modification of the assay described by Noguchi and Higuchi [19]. Samples were collected in EDTA tubes and kept at < 4 °C until the plasma was separated and frozen at −70 °C. Plasma was spiked with...
known amounts of internal standard (mercaptopropionyl glycine), reduced with tri-n-butyl phosphine and then deproteinized with sulphosalicylic acid. The thiol-specific fluorogenic labelling reagent ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate was added to the supernatant, and the sample was neutralized with HCl, washed with a 5% tributyl phosphate solution, filtered and injected on to a reverse-phase HPLC column. Quantification was by measurement of the emission signal of the analyte at 515 nm (excitation 385 nm). The coefficient of variation was < 2%. The results of this assay are expressed as plasma homocyst(e)ine, and represent the sum of free and bound forms of homocysteine, homocysteine and homocysteine/cysteine mixed disulphide. Plasma methionine was measured with an automated amino acid analyser (Beckman 7300; Beckman Instruments, Palo Alto, CA, U.S.A.) using a lithium physiological ion-exchange column system. Quantification was achieved by post-column derivatization with ninhydrin, monitoring UV absorbance with norleucine as an internal standard.

Concentrations of l-arginine and dimethylarginines were determined by HPLC using pre-column derivatization with o-phthalaldehyde by a modification of a previously described method [20]. Samples were spiked with 10 μM homocystine as internal standard, and extracted on C8 solid-phase extraction cartridges (Varian, Harbor City, CA, U.S.A.). The eluates were dried under nitrogen and the residues were dissolved in double-distilled water for HPLC analysis. HPLC was carried out on a Gynkotek liquid chromatography system (Gynkotek, Munich, Germany) consisting of two HPLC pumps with a gradient controller (model M 480 HDG), a spectral fluorescence detector (RF 1002) and an automatic injector (model GINA 160). Samples and standards were incubated for exactly 30 s with the o-phthalaldehyde reagent (5.4 mg/ml in borate buffer, pH 8.5, containing 0.4% 2-mercaptoethanol) before automatic injection into the HPLC system. Chromatographic separation was performed on a C8H8 column (Macherey and Nagel, Düren, Germany) with the fluorescence monitor set at εαx = 340 nm and εαm = 455 nm. Samples were eluted from the column isocratically with 0.96% citric acid/methanol (2:1, v/v, pH 6.8) at a flow rate of 1 ml/min. The coefficients of variation of this method were 5.2% (within-assay) and 5.5% (between-assay); the detection limit of the assay was 0.1 μmol/l.

Calculations and statistical analyses
All analyses were performed by observers blinded to treatment assignment. Brachial artery diameter and blood velocity measurements were analysed using a vascular ultrasound Doppler analysis package provided with the Toshiba machine. For each of three consecutive cardiac cycles, brachial artery diameter (mm) was calculated as the mean of three evenly spaced measurements of the distance from the trailing edge to the leading edge of ultrasonic arterial borders at end-diastole, using electronic calipers on the ultrasound machine. Mean Doppler flow velocity (cm/s) over the entire cardiac cycle was calculated and averaged over three consecutive cardiac cycles. Percentage changes in diameter and velocity were calculated 10 s, 1 min and 2 min after the induction of reactive hyperaemia, and 2, 4 and 6 min after nitroglycerine. In 40 studies performed in healthy subjects with no interventions, dilation in response to reactive hyperaemia was maximal at 2 min after cuff deflation (1 min, 5.6 ± 0.7%; 2 min, 6.2 ± 0.6%), so the 2 min time point is shown in Figures and Tables. Maximum dilation in response to nitroglycerine occurred 6 min after administration (2 min, 6.2 ± 0.7%; 4 min, 14.1 ± 1.4%; 6 min, 14.5 ± 1.5%), so the 6 min time point is shown in Figures and Tables. Our methodology for the assessment of conduit vessel FMD has good within-subject reproducibility, with a correlation coefficient of 0.75 between ten paired studies, and an average coefficient of variability of 13.6% in three subjects studied on four or more occasions.

All data are given as means ± S.E.M. Statistical significance was tested using ANOVA followed by Fisher’s protected least significant difference test. Statistical significance was accepted at P < 0.05.

RESULTS
Subjects were normohomocyst(e)inaemic at baseline. Methionine loading significantly increased plasma methionine and homocyst(e)ine concentrations as compared with placebo (Table 1). The ADMA concentration was also increased significantly (by 69.8 ± 26.2%) after methionine, but not after placebo (17.2 ± 14.2%; P < 0.05 between treatments). There was no association between ADMA and methionine levels. Symmetrical dimethylarginine (SDMA) and l-arginine levels were not changed significantly, resulting in a significantly decreased L-arginine/ADMA ratio after methionine loading (Table 1). There was a significant association between the plasma concentrations of homocyst(e)ine and ADMA in individual subjects (r = 0.48, P < 0.05; Figure 1).

FMD was significantly decreased after methionine (1.54 ± 0.92%) as compared with after placebo (7.08 ± 1.56%; P < 0.05). Flow velocity in the brachial artery during hyperaemia was unchanged (Table 2). Endothelium-independent vasodilation induced by nitroglycerine was also unchanged (Table 2). FMD was inversely correlated with ADMA levels (r = −0.49, P < 0.05; Figure 2) and positively correlated with the l-arginine/ADMA ratio (r = 0.60, P < 0.01; Figure 3).
Table 1  Plasma methionine, homocyst(e)ine, L-arginine and dimethylarginine concentrations
Data are means ± S.E.M. Significance of differences: *P < 0.05 compared with baseline.

<table>
<thead>
<tr>
<th>Concentration (µmol/l)</th>
<th>Methionine</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline 8 h</td>
<td>Baseline 8 h</td>
</tr>
<tr>
<td>Methionine</td>
<td>20.4 ± 1.6</td>
<td>23.0 ± 2.2</td>
</tr>
<tr>
<td>Homocyst(e)ine</td>
<td>6.9 ± 0.6</td>
<td>6.9 ± 0.4</td>
</tr>
<tr>
<td>ADMA</td>
<td>1.39 ± 0.20</td>
<td>1.70 ± 0.21</td>
</tr>
<tr>
<td>SDMA</td>
<td>1.70 ± 0.25</td>
<td>1.89 ± 0.28</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>53.2 ± 7.3</td>
<td>57.1 ± 5.7</td>
</tr>
<tr>
<td>L-Arginine/ADMA ratio</td>
<td>41.2 ± 4.4</td>
<td>35.9 ± 2.6</td>
</tr>
</tbody>
</table>

Table 2  Ultrasonographic determination of FMD and nitroglycerine-induced vasodilation after methionine and placebo
Data are means ± S.E.M. for nine subjects, and are expressed as the percentage change induced by hyperaemia or by nitroglycerine. Significance of difference compared with placebo: *P < 0.05.

<table>
<thead>
<tr>
<th>Change (%)</th>
<th>Methionine</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flow velocity</td>
<td>385 ± 43</td>
<td>411 ± 48</td>
</tr>
<tr>
<td>Diameter</td>
<td>1.54 ± 0.92*</td>
<td>7.08 ± 1.56</td>
</tr>
<tr>
<td>Nitroglycerine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flow velocity</td>
<td>-2.4 ± 4.9</td>
<td>4.6 ± 3.4</td>
</tr>
<tr>
<td>Diameter</td>
<td>16.4 ± 2.0</td>
<td>14.1 ± 1.1</td>
</tr>
</tbody>
</table>

Figure 1  Effects of oral methionine on plasma homocyst(e)ine and ADMA concentrations
Upper panel: changes in plasma homocyst(e)ine and ADMA concentrations for each of nine volunteers before and 8 h after oral methionine loading. Lower panel: relationship between the change in the plasma ADMA concentration and the change in the plasma homocyst(e)ine concentration 8 h after methionine (●) or placebo (○).

Figure 2  Correlation analysis for plasma ADMA concentration and FMD vasodilation in nine healthy human subjects 8 h after methionine (●) or placebo (○)
In contrast, the association between homocyst(e)ine concentration and FMD was much weaker and not statistically significant (r = 0.36).
DISCUSSION

Our main finding is that an acute increase in plasma homocyst(e)ine levels due to oral methionine loading was associated with a significant increase in plasma levels of ADMA, an endogenous inhibitor of NO synthase. Impairment of brachial artery FMD after methionine loading was correlated with ADMA levels, suggesting that ADMA may mediate impaired endothelium-dependent vasodilation during experimental hyperhomocyst(e)inaemia in humans.

Brachial artery FMD is largely dependent upon endothelial NO elaboration [21]. Impairment of NO-mediated vasodilation is a sensitive indicator of the atherosclerotic process, since it occurs early during the development of atherosclerosis [22], and improves early during the regression of atherosclerotic lesions [23,24]. In humans, the degree of endothelial dysfunction is associated with the number of cardiovascular risk factors present in an individual [25]. Therefore endothelium-dependent vasodilation can be regarded as a surrogate marker for atherosclerotic risk.

Controlled studies in humans and in non-human primates have clearly shown an association between hyperhomocyst(e)inaemia and impaired endothelium-dependent, NO-mediated vasodilation. Lentz et al. [1] found that monkeys fed on a diet enriched in methionine and deficient in folate and choline exhibited impaired endothelium-dependent vasodilation in vivo and ex vivo. Acute hyperhomocyst(e)inaemia induced by an oral methionine load impairs endothelium-dependent vasodilation in human subjects [14–16]. In the study by Kanani et al. [16], flow-mediated vasodilation was inversely related to plasma homocyst(e)ine, with \( r = 0.49 \) (\( P < 0.01 \)), in 40 healthy subjects. By contrast, Chambers et al. [26] found no significant correlation between post-load plasma homocyst(e)ine and FMD (\( r = -0.42, P > 0.05 \)) in 17 healthy subjects. Bellamy and co-workers [14] studied 24 healthy humans before and after an acute oral methionine load, and also found that endothelium-dependent vasodilation was significantly impaired simultaneously with an increased plasma homocyst(e)ine concentration. However, that study and our present study found no significant correlation between the change in FMD and the plasma homocyst(e)ine concentration. The discrepancy between studies may be related to the number of subjects tested and to the timing of measurements. This discrepancy also suggests that the link between homocyst(e)ine and endothelium-derived NO may be indirect. This conclusion is further corroborated by a study showing that dietary supplementation with B vitamins did not reverse a pre-existing dysfunction in endothelium-dependent vasodilation in monkeys with diet-induced hyperhomocyst(e)inaemia and hypercholesterolaemia [27].

We recently found an association between homocyst(e)ine levels and plasma concentrations of ADMA in monkeys with diet-induced hyperhomocyst(e)inaemia [9]. In these animals, multivariate analyses revealed that the degree of endothelial dysfunction was associated more closely with ADMA levels than with homocyst(e)ine levels. The synthesis of homocyst(e)ine from methionine involves demethylation of the intermediate, \( S \)-adenosylmethionine [13]. \( S \)-Adenosylmethionine is a ubiquitous methyl group donor, and therefore we speculated that dimethylation of \( L \)-arginine to form ADMA may utilize \( S \)-adenosylmethionine as a methyl group donor. Methionine loading increases substrate availability for methyltransferases, thereby facilitating the synthesis of ADMA. We recently demonstrated that \(^{13}\)C-labelled methyl groups from [methyl \( ^{13}\)C] \( S \)-adenosylmethionine can be incorporated into ADMA by cultured human endothelial cells [28]. In that study, incubation of endothelial cells with methionine induced a concentration-dependent increase in ADMA release, which was inhibited by the \( N \)-methyltransferase inhibitor \( S \)-adenosylhomocysteine [28]. However, because methionine concentrations were higher and the incubation period was longer than the respective plasma concentration and time after oral methionine bolus in the present clinical study, we performed additional incubation experiments with human EAhy.926 endothelial cells. After incubation with \( L \)-methionine at a concentration similar to that reached in the plasma of human volunteers after oral methionine loading for 8 h (i.e. 300 \( \mu \)M), we found that ADMA release was slightly, but significantly, increased compared with the control (control, \( 7.1 \pm 0.4 \) pmol of ADMA/\( \mu \)g of protein; methionine, \( 10.0 \pm 0.6 \) pmol of ADMA/\( \mu \)g of protein; each \( n = 6; P < 0.01 \)). Inhibition of \( S \)-adenosylmethionine-
dependent methyltransferase also reduced baseline ADMA formation and the low-density-lipoprotein-cholesterol-induced increase in ADMA release in cultured human endothelial cells [28]. N-Methyltransferases present in human endothelium specifically asymmetrically methylate L-arginine residues, resulting in the release of ADMA and Nω-monomethyl-L-arginine, but not SDMA [28]. Thus there is strong experimental evidence that this mechanism (i.e. elevation of ADMA formation due to increased N-methyltransferase activity) also plays a role in endothelial dysfunction after acute methionine loading in human subjects.

Another possible explanation for the selective increase in ADMA levels after methionine loading may be decreased activity of dimethylarginine dimethylamino-hydrolase (DDAH), an enzyme which metabolizes ADMA, but not SDMA, to citrulline and dimethylamine [29]. However, neither methionine nor homocysteine modifies DDAH activity in cultured endothelial cells in vitro (R. H. Böger, unpublished work), making it improbable that this is the molecular mechanism underlying our present observations.

The increase in the plasma concentration of ADMA evoked by oral methionine loading in the present study (1.4 to 2.0 μmol/l) may appear rather limited and insignificant at first sight. However, there is evidence that such small differences in plasma ADMA concentrations can induce significant differences in endothelial function [10]. Moreover, experimental data suggest that ADMA levels within endothelial cells (i.e. at the site of NO production by endothelial NO synthase) may be much higher than those measured extracellularly [30,31]. Additional evidence indicates that inhibition of DDAH activity, which increases endogenous ADMA levels within cells, inhibits endothelium-dependent relaxation in isolated arterial rings [32], suggesting that ADMA is involved in the regulation of NO synthase activity.

Experimental data suggest that homocyst(e)ine directly alters the biological availability of endothelial NO by a mechanism involving oxidative inactivation of this molecule [33]. Upchurch et al. [34] found that homocysteine impairs the ability of cultured bovine aortic endothelial cells to detoxify hydrogen peroxide, thus rendering NO more susceptible to oxidative inactivation. Our group has shown that endothelial dysfunction caused by methionine loading can be reversed by administration of an antioxidant (ascorbic acid) [16]. However, other investigators failed to show increased oxidative stress during acute or medium-term supplementation with oral methionine [35].

Our present finding that ADMA is involved in homocyst(e)ine-induced endothelial dysfunction is not in conflict with the observations of elevated oxidative stress during experimental hyperhomocyst(e)inaemia as cited above. It is known that inhibition of endothelial NO synthase shifts the balance between NO and O2·− in favour of oxidative stress [36]. Böger et al. [31] reported that incubation of human endothelial cells with ADMA increases endothelial superoxide release and leads to the activation of redox-regulated genes. Lin et al. [37] extended this finding by showing that inhibition of the metabolism of endogenous AMDA by the enzyme DDAH increased the ADMA concentration in conditioned media and also enhanced endothelial superoxide release. Finally, Xiong et al. [38] showed that elevation of dimethylarginine levels in cholesterol-fed rabbits was associated with lipid peroxidation.

Our present findings, therefore, suggest that L-arginine may function as a methyl group acceptor during the demethylation of methionine to homocyst(e)ine, resulting in increased formation of ADMA. ADMA acts as an endogenous inhibitor of endothelial NO synthase [8], and an elevated ADMA concentration constitutes a risk factor for endothelial dysfunction in humans [10]. Thus our findings suggest a novel mechanism for impaired endothelial function associated with hyperhomocyst(e)inaemia.

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