Effects of acute methionine loading and vitamin C on endogenous fibrinolysis, endothelium-dependent vasomotion and platelet aggregation

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ABSTRACT

We assessed forearm blood flow and plasma fibrinolytic factors in eight healthy males who received unilateral brachial artery infusions of the endothelium-dependent vasodilator, substance P, and the endothelium-independent vasodilator, sodium nitroprusside. These measurements, together with platelet aggregation studies, were performed on four occasions after double-blind randomized ingestion of placebo, methionine (0.1 mg/kg), vitamin C (2 g) and methionine plus vitamin C. Blood flow and platelet aggregation responses were unaffected by methionine loading. Substance P caused dose-dependent increases in plasma tissue plasminogen activator (t-PA) antigen (from 3.0 + 0.1 to 4.7 + 0.4 ng/ml; \( P < 0.001 \)) and activity (from 1.2 + 0.2 to 4.2 + 0.4 i.u./ml; \( P < 0.001 \)), which were augmented during acute methionine loading (4.7 + 0.4 to 5.6 + 0.5 ng/ml and 4.2 + 0.4 to 5.5 + 0.9 i.u./ml respectively; \( P < 0.05 \)). Moreover, the estimated net release of t-PA was enhanced during methionine loading (two-way ANOVA; \( P = 0.02 \)), but this was unaffected by vitamin C supplementation. We conclude that, in the absence of alterations in endothelium-dependent vasomotion or platelet aggregation, substance P-induced t-PA release is enhanced following methionine loading. This suggests that the acute endogenous fibrinolytic capacity is augmented during acute hyperhomocysteinaemia in healthy humans via an oxidation-independent mechanism.

INTRODUCTION

Elevated plasma homocysteine concentrations are an independent risk factor for myocardial infarction [1], are associated with thrombophilia, and confer a more than 20-fold increased risk of coronary artery disease [2]. Severe hyperhomocysteinaemia is rare, but moderate hyperhomocysteinaemia is more common [3–5] and is present in ~30% of patients with premature coronary artery disease [2]. In contrast with serum lipid fractions, homocysteine concentrations are correlated only weakly with the extent of coronary atheroma [6], suggesting that the association with coronary events is more likely to represent thrombogenicity or plaque rupture rather than atherogenicity.

It has been recognized for over 20 years that elevated plasma concentrations of homocysteine cause endothelial cell injury and denudation in animal models [7,8]. However, more recent studies have confirmed that endothelial dysfunction is also present in human subjects with hyperhomocysteinaemia [9–12], and that this may be reversed by lowering plasma homocysteine concentrations using pyridoxine and folate therapy [13]. The mechanism whereby hyperhomocysteinaemia produces

Key words: blood flow, endothelium, hyperhomocysteinaemia, plasminogen activators, platelet aggregation.

Abbreviations: t-PA, tissue plasminogen activator; PAI, plasminogen activator inhibitor; a.u., arbitrary units.

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endothelial dysfunction may, in part, relate to oxidative stress and superoxide generation [14]. Indeed, acute antioxidant administration using vitamin C appears to reverse the impairment of flow-associated vasodilatation induced by methionine loading in healthy volunteers [15].

The fibrinolytic factor tissue plasminogen activator (t-PA) and its inhibitor, plasminogen activator inhibitor type 1 (PAI-1), are potentially important endothelium-derived mediators that are intimately linked to the risk of thrombosis. The time course of t-PA release is important, since clot dissolution is much more effective if t-PA is incorporated during clot formation rather than following completion [16,17]. However, the capacity of endothelial cells to release t-PA from intracellular storage pools, and the rapidity with which it can be mobilized, may not necessarily be reflected in the basal circulating plasma concentrations of t-PA antigen or its activity [18,19]. We have shown, using bilateral forearm venous occlusion plethysmography and unilateral brachial artery infusions, that the forearm release of t-PA can be determined in vivo in humans [20]. This model permits a more precise pharmacological stimulus to be applied to the endothelium in a well defined and reproducible manner. Moreover, on applying this model to healthy subjects with a smoking habit, we have shown that, despite higher basal plasma antigen concentrations, cigarette smokers have a markedly impaired capacity to release t-PA acutely [18].

In a longitudinal study of stroke patients, plasma t-PA concentrations were found to be an independent discriminator, and were correlated directly with plasma homocysteine concentrations [21]. Moreover, methionine loading causes more pronounced alterations in basal fibrinolytic parameters of patients with premature vascular disease [22]. Although it would appear that hyperhomocysteinaemia may be associated with alterations in endogenous fibrinolysis, it is not known whether changes in plasma homocysteine concentrations can influence the acute release of t-PA from the endothelium.

In addition to procoagulant effects on the vessel wall, hyperhomocysteinaemia may induce vascular occlusion through platelet actions [23,24]. Animal studies [23,25] indicate that hyperhomocysteinaemia enhances platelet aggregation. However, the effects of acute elevations of plasma homocysteine concentrations on the activity of platelets in humans are currently unknown.

We hypothesized that experimental hyperhomocysteinaemia, produced by methionine loading, would impair substance P-induced forearm vasodilatation and t-PA release, and enhance platelet aggregation. Moreover, given the potential role of oxidative stress, we further hypothesized that vitamin C supplementation would reverse these potential derangements in endothelial and platelet function. Therefore the aims of the present study were to examine the effects of increased plasma homocysteine concentrations, following an oral methionine load, on acute endogenous t-PA release, endothelium-dependent vasomotion and platelet aggregation, and to determine the influence of vitamin C supplementation on these responses.

METHODS
Subjects
Eight healthy male non-smokers, aged between 20 and 42 (mean 30) years, participated in a four-phase study, which was undertaken with the approval of the local research ethics committee and in accordance with the Declaration of Helsinki (1989) of the World Medical Association. The written informed consent of each subject was obtained before entry into the study. Volunteers were normotensive, with normal serum cholesterol, folate and vitamin B<sub>12</sub> levels. None of the subjects received vasoactive or non-steroidal anti-inflammatory drugs in the week before each phase of the study, and all abstained from alcohol for 24 h, and from caffeine-containing foods for at least 12 h, before each study. All studies were carried out in a quiet, temperature-controlled room maintained at 22–24 °C.

Drugs
Pharmaceutical-grade substance P (Clinalfa AG, Läufelfingen, Switzerland) and sodium nitroprusside (Nipride; Roche, Welwyn Garden City, U.K.) were administered following dissolution in 0.9 % saline (Baxter Healthcare Ltd, Thetford, Norfolk, U.K.). In order to maintain blinding, methionine (0.1 mg/kg; Evans Medical Ltd, Leatherhead, Surrey, U.K.) [12], vitamin C (2 g; Redoxen; Roche) and placebo were administered following dissolution in an orange-flavoured drink containing no vitamin C.

Intra-arterial administration
The brachial artery of the non-dominant arm was cannulated with a 27-standard wire gauge steel needle (Cooper’s Needle Works Ltd, Birmingham, U.K.) under local anaesthesia, and attached to a 16 gauge epidural catheter (Portex Ltd, Kings Langley, Herts., U.K.) local anaesthesia, and attached to a 16 gauge epidural catheter (Portex Ltd, Hythe, Kent, U.K.). Patency was maintained by infusion of saline via an IVAC P1000 syringe pump (IVAC Ltd, Basingstoke, Hants., U.K.). The total rate of intra-arterial infusion was maintained constant throughout all studies at 1 ml/min.

Forearm blood flow and haemodynamics
Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic
strain gauges applied to the widest part of the forearm [26]. During measurement periods, the hands were excluded from the circulation by rapid inflation of wrist cuffs to a pressure of 220 mmHg using E20 Rapid Cuff Inflators (D. E. Hokanson Inc., Washington, DC, U.S.A.). Upper-arm cuffs were inflated intermittently to 40 mmHg for 10 s in every 15 s to achieve venous occlusion and obtain plethysmographic recordings. Analogue voltage output from an EC-4 Strain Gauge Plethysmograph (D. E. Hokanson Inc.) was processed by a MacLab® analogue-to-digital converter and Chart® v3.3.8 software (AD Instruments Ltd, Castle Hill, NSW, Australia) and recorded on to a MacIntosh Classic II computer (Apple Computers Inc.). Calibration was achieved using the internal standard of the plethysmograph.

Blood pressure and heart rate were monitored in the non-infused arm at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer (Takeda UA 751; Takeda Medical Inc., Tokyo, Japan) [27].

Assays
t-PA and and PAI-1
Venous cannulae (17G) were inserted into large subcutaneous veins of the antecubital fossa in both arms. A 10 ml blood sample was withdrawn simultaneously from each arm and collected into acidified buffered citrate (Biopool®; Stabilyte, Umeå, Sweden; for t-PA assays) and citrate (Monovette®; Sarstedt, Nümbrecht, Germany; for PAI-1 assays) tubes, and kept on ice before being centrifuged at 2000 g for 30 min at 4 °C. Platelet-free plasma was decanted and stored at −80 °C before assay [28].

Plasma PAI and t-PA antigen concentrations were determined using ELISAs: Coaliza* PAI-1 [29] and Coaliza* t-PA [30] (Chromogenix AB, Mölndal, Sweden) respectively. Plasma PAI and t-PA activities were determined by a photometric method: Coatest* PAI-1 [31] and Coatest* t-PA [32] (Chromogenix AB). Intra-assay coefficients of variation were 7.0 % and 5.5 % respectively for t-PA and PAI-1 antigen, and 4.0 % and 2.4 % respectively for activity. Inter-assay coefficients of variation were 4.0 %, 7.3 %, 4.0 % and 7.6 % respectively. The sensitivities of the assays were 0.5 ng/ml, 2.5 ng/ml, 0.10 i.u./ml and 5 arbitrary units (a.u.)/ml respectively. The haematocrit was determined from the infused forearm at baseline and during infusion of 8 pmol of substance P/min.

Homocysteine and vitamin C
Plasma total homocysteine concentrations were measured, as described previously [33], in blood collected in lithium/heparin (Monovette*). In brief, following addition of 2-mercaptoethanolamine and reduction with tri-n-butylphosphine, plasma was deproteinized using 10% trichloroacetic acid and derivatized with 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonic acid at 60 °C for 1 h. Derivatized thiols were separated by HPLC (C18 columns; Millipore, Milford, MA, U.S.A.; mobile phase 6% acetonitrile in 0.1 mol/l KH2PO4 adjusted to pH 2.15 with H3PO4; flow rate 0.8 ml/min) and detected in a fluorescence detector (Perkin Elmer LS30; excitation 385 nm, emission 513 nm). Chromatograms were analysed using computer software (JCL 6000 Chromatography Data System; Jones Chromatography, Lakewood, CO, U.S.A.) and expressed as the quotient of the homocysteine peak area (retention time 5.3 min) and the internal standard peak area (retention time 3.3 min). The peak ratio showed a linear relationship with homocysteine concentration in spiked plasma samples (< 2.5 to > 40 μmol/l; \( r^2 = 0.997 \)). Inter- and intra-assay coefficients of variation were < 12% and < 8% respectively.

Blood samples in lithium/heparin (Monovette*) were centrifuged immediately, and 500 μl of plasma was separated before being rapidly snap-frozen following addition of 500 μl of 5% metaphosphoric acid. Plasma vitamin C concentrations were determined using an enzymic colorimetric method, as previously described [34] (Cobas Bio centrifugal analyser equipped with a fluorescence attachment).

Platelet aggregometry
A 30 ml sample of blood was collected into trisodium citrate tubes (Monovette*) and immediately centrifuged at 120 g for 10 min to obtain platelet-rich plasma, which was aspirated and pre-warmed to 37 °C. Aggregation studies were performed, 30–40 min after blood sampling, using a standard optical technique (Chronolog Ca560 aggregometer; Labmedics, Stockport, U.K.) as described previously [35]. The maximum aggregation attained within 7 min of the addition of each ADP concentration (1–8 μmol/l) was recorded, and expressed as a percentage of the response to 8 μmol/l ADP. Measurement of platelet counts and haematocrit were performed using an automated Coulter counter (ACT.8 Coulter Counter; Beckman-Coulter, High Wycombe, U.K.).

Study design
All subjects attended at 08.00 hours on each of four study days at least 1 week apart, and participated in each of the four study phases: double placebo, methionine plus placebo, vitamin C plus placebo, and methionine plus vitamin C. Blood samples were obtained for the measurement of plasma homocysteine concentrations and platelet aggregation before and 6 h after the ingestion of double placebo, methionine plus placebo, vitamin C plus placebo or methionine plus vitamin C, administered in a double-
Table 1. Absolute blood flow in the infused and non-infused forearms during substance P infusion: endothelium-dependent vasomotion

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Blood flow (ml·min⁻¹·100 ml⁻¹)</th>
<th>Infused arm</th>
<th>Non-infused arm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 2 4 8 P</td>
<td>0 2 4 8</td>
</tr>
<tr>
<td>Placebo supplementation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>5.8 ± 1.0 13.9 ± 0.9</td>
<td>17.4 ± 1.2</td>
<td>22.1 ± 1.9</td>
</tr>
<tr>
<td>Methionine</td>
<td>4.9 ± 0.5 12.8 ± 1.4</td>
<td>14.9 ± 1.3</td>
<td>17.0 ± 1.8</td>
</tr>
<tr>
<td>Vitamin C supplementation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>4.6 ± 0.8 12.2 ± 2.1</td>
<td>14.7 ± 2.2</td>
<td>18.2 ± 2.8</td>
</tr>
<tr>
<td>Methionine</td>
<td>4.7 ± 0.9 12.3 ± 1.5</td>
<td>15.6 ± 2.3</td>
<td>20.3 ± 3.2</td>
</tr>
</tbody>
</table>

Data analysis and statistics

The study population size, based on power calculations derived from previous studies [20], gives 90% power of detecting a 21% difference in t-PA release at a significance level of 5%. Coefficients of repeatability [37] for plasma concentrations of t-PA antigen and activity during substance P infusion at 8 pmol/min are 1.6 ng/ml and 1.4 i.u./ml respectively (results not shown).

Plethysmographic and aggregometry data were extracted from the Chart™ data files. Forearm blood flows were calculated for individual venous occlusion cuff inflations by use of a template spreadsheet (Excel v5.0; Microsoft Corp.). Recordings from the first 60 s after wrist cuff inflation were not used, because of the variability in blood flow this causes. Usually, the last five flow recordings in each 3 min measurement period were calculated and averaged for each arm. Estimated net release of t-PA activity and antigen was defined previously [20] as the product of the infused forearm plasma flow (based on the mean haematocrit (Hct) and the infused forearm blood flow (FBF)) and the concentration difference between the infused ([t-PA]inf) and non-infused ([t-PA]non-inf) arms:

Estimated net t-PA release = FBF × (1 − Hct) × ([t-PA]inf − [t-PA]non-inf)

Data were examined, where appropriate, by two-way ANOVA for repeated measures and two-tailed paired Student’s t-test using Excel v5.0 (Microsoft). All results are expressed as means ± S.E.M. Statistical significance was taken at the 5% level.

RESULTS

There were no significant differences in baseline blood pressure, heart rate, haematocrit (results not shown) or forearm blood flow between the four study days (Tables 1 and 2). On each of the study days, blood pressure, heart rate, haematocrit (results not shown) and non-infused forearm blood flow (Tables 1 and 2) did not change during the study. However, plasma homocysteine concentrations (Figure 1) were elevated in all subjects following methionine plus placebo or methionine plus vitamin C (P = 0.01; paired t-test), but not after double placebo or placebo plus vitamin C. Plasma vitamin C concentrations increased after vitamin C plus placebo or vitamin C plus methionine (P < 0.001), but not after double placebo or placebo plus methionine (Figure 1).
Table 2  Absolute blood flow in the infused and non-infused forearms during sodium nitroprusside infusion: endothelium-independent vasomotion

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SNP (µg/min)</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo supplementation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>4.2 ± 0.3</td>
<td>16.9 ± 3.9</td>
<td>20.2 ± 4.1</td>
<td>20.7 ± 3.1</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>4.1 ± 0.4</td>
<td>10.8 ± 2.7</td>
<td>14.0 ± 1.1</td>
<td>16.9 ± 1.4</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Vitamin C supplementation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>3.6 ± 0.4</td>
<td>11.5 ± 2.3</td>
<td>15.9 ± 2.9</td>
<td>20.6 ± 4.4</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>4.1 ± 0.4</td>
<td>13.2 ± 3.3</td>
<td>17.3 ± 3.9</td>
<td>20.8 ± 3.4</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
</tbody>
</table>

**Endothelium-dependent and -independent vasomotion**

Sodium nitroprusside and substance P caused dose-dependent increases in blood flow of the infused forearm (ANOVA: \( P < 0.001 \) for all; Tables 1 and 2) on all four study days. The blood flow responses to both sodium nitroprusside and substance P were similar on each of the study days, with no significant differences in the magnitude of the responses. There was no time order effect of the blood flow responses to substance P or sodium nitroprusside.

**Endogenous fibrinolysis**

There were no significant differences between baseline plasma t-PA and PAI-1 concentrations on each of the four study days. Substance P caused dose-dependent increases in plasma t-PA antigen concentration and activity in the infused, but not the non-infused, forearm on each of the four study days (Table 3). Moreover, the concentration difference between the infused and non-infused forearms also increased significantly with substance P infusion (Table 3). Estimated net release of t-PA antigen and activity demonstrated dose-dependent increases (ANOVA: \( P < 0.001 \) for all; Figure 2), which were unaffected by co-administration of vitamin C. Substance P-induced increases in t-PA plasma concentrations and forearm release were significantly greater following methionine loading than after placebo (Table 3 and Figure 2).

At 6 h after administration of double placebo, methionine plus placebo, vitamin C plus placebo and methionine plus vitamin C, basal plasma PAI-1 antigen concentrations were 20±5, 18±3, 20±2 and 16±2 ng/ml respectively, and PAI-1 activities were 8±1, 7±2, 8±1 and 7±1 a.u./ml respectively. There were no significant differences between the study days.

**Platelet aggregation**

There were no differences in platelet counts before or 6 h after the administration of double placebo ([(442±27)×10^9/l and (415±17)×10^9/l respectively]) or methionine plus placebo ([429±32]×10^9/l and (411±25)×10^9/l respectively). There were no significant changes in the concentration–response curves for ADP-induced platelet aggregation in platelet-rich plasma 6 h
after oral administration of double placebo or methionine plus placebo (Figure 3). Maximal aggregation (theoretical maximum 80 mV) induced by 8 μM ADP was 68 ± 4 and 65 ± 3 mV following placebo and methionine administration respectively.

<table>
<thead>
<tr>
<th>SP infusion (pmol/min) . . .</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo supplementation</td>
<td></td>
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<td></td>
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<tr>
<td>t-PA antigen (ng/ml)</td>
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</tr>
<tr>
<td>Infused arm</td>
<td>3.0 ± 0.1</td>
<td>3.5 ± 0.2</td>
<td>3.9 ± 0.2</td>
<td>4.7 ± 0.4</td>
<td>≤ 0.001</td>
</tr>
<tr>
<td>Non-infused arm</td>
<td>3.1 ± 0.1</td>
<td>3.4 ± 0.2</td>
<td>3.4 ± 0.2</td>
<td>3.5 ± 0.2 NS</td>
<td></td>
</tr>
<tr>
<td>Forearm difference</td>
<td>-0.1 ± 0.1</td>
<td>0.1 ± 0.2</td>
<td>0.5 ± 0.2</td>
<td>1.2 ± 0.3</td>
<td>≤ 0.001</td>
</tr>
<tr>
<td>Net release (ng·min⁻¹·100 ml⁻¹)</td>
<td>-0.4 ± 0.2</td>
<td>0.8 ± 1.9</td>
<td>5.8 ± 20.15</td>
<td>8.4 ± 0.4</td>
<td>≤ 0.001</td>
</tr>
<tr>
<td>t-PA activity (i.u./ml)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Infused arm</td>
<td>1.2 ± 0.2</td>
<td>2.1 ± 0.4</td>
<td>2.6 ± 0.4</td>
<td>4.2 ± 0.4</td>
<td>≤ 0.001</td>
</tr>
<tr>
<td>Non-infused arm</td>
<td>1.5 ± 0.3</td>
<td>1.7 ± 0.5</td>
<td>1.5 ± 0.2</td>
<td>1.9 ± 0.2 NS</td>
<td></td>
</tr>
<tr>
<td>Forearm difference</td>
<td>-0.2 ± 0.1</td>
<td>0.4 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>2.3 ± 0.4</td>
<td>≤ 0.001</td>
</tr>
<tr>
<td>Net release (i.u.·min⁻¹·100 ml⁻¹)</td>
<td>-1.0 ± 0.7</td>
<td>3.3 ± 1.9</td>
<td>11.16 ± 24.31 ± 7.1</td>
<td>≤ 0.001</td>
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</tr>
<tr>
<td>Vitamin C supplementation</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>t-PA antigen (ng/ml)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Infused arm</td>
<td>3.1 ± 0.2</td>
<td>3.6 ± 0.2</td>
<td>3.9 ± 0.3</td>
<td>4.3 ± 0.4</td>
<td>≤ 0.05</td>
</tr>
<tr>
<td>Non-infused arm</td>
<td>3.4 ± 0.2</td>
<td>3.2 ± 0.2</td>
<td>3.4 ± 0.2</td>
<td>3.6 ± 0.3 NS</td>
<td></td>
</tr>
<tr>
<td>Forearm difference</td>
<td>-0.3 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.5 ± 0.3</td>
<td>0.7 ± 0.3</td>
<td>≤ 0.05</td>
</tr>
<tr>
<td>Net release (ng·min⁻¹·100 ml⁻¹)</td>
<td>-0.8 ± 0.2</td>
<td>2.2 ± 1.0</td>
<td>5.1 ± 2.8</td>
<td>8.9 ± 4.0</td>
<td>≤ 0.05</td>
</tr>
<tr>
<td>t-PA activity (i.u./ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infused arm</td>
<td>1.3 ± 0.3</td>
<td>2.2 ± 0.3</td>
<td>2.9 ± 0.5</td>
<td>4.0 ± 0.6</td>
<td>≤ 0.001</td>
</tr>
<tr>
<td>Non-infused arm</td>
<td>1.5 ± 0.3</td>
<td>1.5 ± 0.3</td>
<td>1.4 ± 0.3</td>
<td>1.8 ± 0.3 NS</td>
<td></td>
</tr>
<tr>
<td>Forearm difference</td>
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<td>0.7 ± 0.2</td>
<td>1.5 ± 0.5</td>
<td>2.3 ± 0.6</td>
<td>≤ 0.001</td>
</tr>
<tr>
<td>Net release (i.u.·min⁻¹·100 ml⁻¹)</td>
<td>-0.5 ± 0.2</td>
<td>5.6 ± 2.4</td>
<td>15.7 ± 5.8</td>
<td>29.7 ± 9.1</td>
<td>≤ 0.01</td>
</tr>
</tbody>
</table>

**DISCUSSION**

In the absence of alterations in endothelium-dependent vasomotion and platelet aggregation, we report, for the first time, a modest enhancement of substance P-induced t-PA release following methionine loading. This effect does not appear to be mediated through oxidative stress, since co-administration of vitamin C had no effect on this response. Thus it would appear that the acute endogenous fibrinolytic capacity is augmented during acute hyperhomocysteinaemia in healthy humans.

The importance of endogenous t-PA release is exemplified by the high rate of spontaneous reperfusion in the infarct-related artery after acute myocardial infarction; this occurs in around 30% of patients within the first 12 h [38-40]. It would be anticipated that high plasma t-PA concentrations should protect against subsequent coronary events. However, in epidemiological studies of patients with ischaemic heart disease [41,42], and in prospective studies in healthy populations [43,44], higher total plasma t-PA (antigen) concentrations positively and independently predict future coronary events. This may, in part, be explained by the concomitant elevation of PAI-1, which forms a complex with t-PA and thereby causes an overall decrease in free t-PA ‘activity’ [45,46]. It is the free and unbound t-PA which is physiologically active and leads to endogenous fibrinolysis. Moreover, in the Northwick Park Heart Study [46] small changes (25%) in fibrinolytic activity were associated with significant increases in the risk of sustaining a myocardial infarction or sudden cardiac death.

The findings of the present study suggest that acute hyperhomocysteinaemia potentiates stimulated t-PA
release. This may be a consequence of acute endothelial cell injury or perturbation, which results in an enhanced response on further provocation. However, chronic hyperhomocysteinaemia may have very different effects, which may include chronic endothelial injury, desensitization and a reduced t-PA response. The results of the present study cannot be extrapolated to chronic hyperhomocysteinaemia, and this requires further careful investigation.

In general, platelet aggregation is sensitive to defects in platelet function rather than to increased platelet activity. However, previous animal studies have indicated that acute methionine loading can enhance platelet aggregation in response to ADP and thrombin [25]. In the light of these findings, we felt that assessment of platelet aggregation could be a useful indicator in our clinical study. However, we have found no evidence of an effect of methionine loading on platelet aggregation in response to ADP. This suggests that hyperhomocysteinaemia is unlikely to alter platelet activity in humans.

In contrast with previous workers [12,15], we have not found an impairment of endothelium-dependent vaso-motion in acute hyperhomocysteinaemia. This is despite the use of a comparable regimen of oral methionine loading and a large rise in plasma homocysteine concentrations. However, previous studies [12,15] have used flow-associated dilatation as a non-invasive method of assessing conduit artery endothelial function. In the present study, we have predominantly assessed the function of endothelium within the resistance vessel bed, in terms of the capacity both to release t-PA and to increase forearm blood flow. Conduit artery and microvascular endothelial cells have distinct phenotypic differences, and this may contribute to the apparent disparity in the responses to the same acute insult. Moreover, responses to mechanical rather than pharmacological stimuli may also differ.

Recently, two studies [47,48] have assessed endothelium-dependent resistance vessel function following acute methionine loading. Using the endothelium-dependent vasodilator acetylcholine, these studies have demonstrated either an impairment of [47], or no effect on [48], forearm resistance vessel endothelial

Figure 2  Estimated net release of t-PA during incremental substance P infusion

Upper panel: ■, placebo plus placebo or vitamin C (n = 16); ○, methionine plus placebo or vitamin C (n = 16). Lower panel: ○, double placebo (n = 8); ●, methionine plus placebo (n = 8). Significance of differences: one-way ANOVA, *P < 0.001 for all (substance P dose–response); two-way ANOVA, *P = 0.02, †P = 0.06 (placebo compared with methionine).

Figure 3  Platelet aggregation in response to incremental doses of ADP at baseline (●) and 6 h post-ingestion of double placebo or methionine plus placebo (△)
function. These apparently contradictory findings may, in part, reflect the differing study designs. Using a study design similar to that of Kanani and colleagues [47], we achieved similar increases in plasma homocysteine concentrations with acute methionine loading, but failed to detect a significant difference in endothelium-dependent vasodilation using substance P. This may be explained by the differing signal transduction pathways involved in acetylcholine- and substance P-induced vasodilation [49,50]. Indeed, previous studies assessing endothelial function, using both substance P and acetylcholine administration, have documented either concordant [51,52] or discordant [53,54] responses that are, in part, dependent upon the disease processes under investigation. Moreover, we chose to use substance P as an endothelial cell stimulant because acetylcholine does not induce the acute release of t-PA in the human forearm [55]. These observations highlight the important differences between the various agents used to stimulate the endothelium, as well as the differing manifestations of endothelial dysfunction that are dependent on the nature of the underlying cellular injury.

In summary, we have found a modest and selective enhancement of substance P-induced t-PA release following methionine loading. It would appear that the acute endogenous fibrinolytic capacity is augmented during acute hyperhomocysteinaemia in healthy humans via an oxidation-independent mechanism.

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