Asymmetrical dimethylarginine regulates endothelial function in methionine-induced but not in chronic homocystinemia in humans: effect of oxidative stress and proinflammatory cytokines\textsuperscript{1−4}

Charalambos Antoniades, Dimitris Tousoulis, Kyriakoula Marinou, Carmen Vasiiliadou, Costantinos Tentolouris, George Bouras, Christos Pitsavos, and Christodoulos Stefanadis

ABSTRACT

Background: Homocystinemia is a metabolic abnormality associated with endothelial dysfunction and increased cardiovascular disease risk. The underlying mechanisms of these effects, however, are obscure.

Objective: We examined the effect of asymmetrical dimethylarginine (ADMA) on endothelial dysfunction in methionine-induced and chronic homocystinemia and evaluated the regulatory role of oxidative stress and proinflammatory cytokines on the release of ADMA.

Design: In this double-blind, placebo-controlled parallel group study, 30 subjects of both sexes (15 with homocystinemia and 15 healthy controls) underwent methionine loading, with simultaneous administration of a combination of vitamin C (2 g) plus \( \alpha \)-tocopherol (800 IU) or placebo. Endothelial function in forearm resistance vessels and concentrations of ADMA, oxidized LDL, and proinflammatory cytokines were determined at baseline and 4 h after methionine loading.

Results: Both chronic and methionine-induced homocystinemia were associated with increased oxidized LDL (\( P < 0.01 \)), higher expression of the proinflammatory cytokine interleukin 6 (\( P < 0.05 \)), and endothelial dysfunction (\( P < 0.01 \)). Although ADMA rapidly increased in acute homocystinemia (\( P < 0.01 \)) and was correlated with forearm hyperemic response at 4 h after methionine loading (\( r = -0.722, P = 0.0001 \)), it was not higher in subjects with high versus low fasting homocysteine. High-dose antioxidant treatment prevented methionine-induced elevation of oxidized LDL and interleukin 6 but failed to prevent the increase in ADMA or endothelial dysfunction.

Conclusions: Both chronic and methionine-induced homocystinemia are characterized by increased oxidative stress and proinflammatory cytokines, which may contribute to the development of endothelial dysfunction. However, the ADMA pathway is activated only in acute homocystinemia by mechanisms not mediated by oxidized LDL or proinflammatory stimuli.


KEY WORDS Vitamins, endothelium, asymmetrical dimethylarginine, ADMA, atherosclerosis, homocysteine, inflammation

INTRODUCTION

Homocystinemia is associated with increased cardiovascular disease risk in humans (1), and even a moderate increase in fasting homocysteine (fHcy) concentrations (\( \geq 10 \) \( \mu \text{mol/L} \)) is associated with an increased risk of atherosclerosis (2). Although endothelial dysfunction is a major link between homocystinemia and atherogenesis (3), the underlying mechanisms of this relation remain obscure.

Increased oxidative stress has been proposed as the main mechanism underlying endothelial dysfunction in both chronic (4) and acute (5, 6) methionine-induced homocystinemia, but this hypothesis was recently questioned (7), and other mechanisms, such as an increase in the endogenous endothelial nitric oxide synthase (eNOS) inhibitor asymmetrical dimethylarginine (ADMA), have been proposed (8). Methionine-induced homocystinemia increases ADMA concentrations by triggering protein arginine methylation (9), and evidence from in vitro studies suggests that the increase in reactive oxygen species accompanying homocystinemia may impair the enzymatic systems participating in ADMA degradation, thus increasing its plasma concentrations (10). However, it is unclear whether oxidative stress is a connective link between homocysteine and ADMA in humans.

Homocystinemia has been associated with increased concentrations of proinflammatory cytokines (11), which lead to both decreased nitric oxide production (12) and increased expression of adhesion molecules on vascular endothelium (13). Furthermore, proinflammatory cytokines play a regulatory role in ADMA synthesis. Tumor necrosis factor \( \alpha \) (TNF-\( \alpha \)) decreases the activity of dimethylarginine dimethylaminohydrolase (DDAH) (10), the enzyme responsible for ADMA degradation, which thus affects eNOS function and nitric oxide synthesis (14). Although the inflammatory component of atherogenesis in homocystinemia is well described (15), it is unclear whether oxidative stress mediates the homocysteine-related alterations in the inflammatory process, and the role of cytokines as stimuli for ADMA synthesis in methionine-induced homocystinemia in humans is unknown.

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Demosgraphic characteristics and lipid profile of the participants

<table>
<thead>
<tr>
<th></th>
<th>High fasting homocysteine</th>
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<tbody>
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<td>Vitamin group</td>
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<tr>
<td>Sex (M/F)</td>
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<tr>
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</tr>
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<td>Fasting homocysteine (µmol/L)</td>
<td>16.5 (13.4–46.3)</td>
<td>24.9 (14.5–33.5)</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>164.9 ± 20.9</td>
<td>163.9 ± 15.2</td>
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<tr>
<td>Triglycerols (mg/dL)</td>
<td>68.0 ± 10.0</td>
<td>67.2 ± 10.5</td>
</tr>
<tr>
<td>HDL (mg/dL)</td>
<td>44.4 ± 5.06</td>
<td>44.5 ± 6.3</td>
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<tr>
<td>Glucose (mg/dL)</td>
<td>79.0 ± 3.2</td>
<td>74.0 ± 10.9</td>
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<tr>
<td>Creatinine clearance (mL/min)</td>
<td>109.5 ± 5.1</td>
<td>115.9 ± 7.1</td>
</tr>
</tbody>
</table>

1 Normally distributed variables are expressed as ˣ±SEM, and comparisons were performed by using one-way ANOVA for multiple comparisons. Non-normally distributed variables are expressed as median (25th–75th percentile), and comparisons were performed by Kruskal–Wallis test. The 4 groups did not differ significantly in any variable except homocysteine (P < 0.0001 between those with high and those with low fasting homocysteine).

2 Calculated by using the Cockcroft-Gault formula.

We examined the contributions of oxidative stress and ADMA to the development of endothelial dysfunction in methionine-induced and chronic homocysteinemia. Furthermore, we evaluated the regulatory role of oxidative stress in the expression of proinflammatory cytokines in both methionine-induced and chronic homocysteinemia, and we examined the hypothesis that oxidized LDL, directly or through an increase in inflammatory cytokines, triggers the synthesis of ADMA in human homocysteinemia.

SUBJECTS AND METHODS

Patient characteristics and protocol design

In this double-blind, placebo-controlled, parallel group study, 30 young subjects with no classic risk factors for atherosclerosis were recruited (Table 1). Fifteen participants had fHcy concentrations ≥12 µmol/L [median (25th–75th percentile): 23.7 (13.77–36.81) µmol/L] and were defined as subjects with high fHcy. Fifteen participants had fHcy concentrations <12 µmol/L [median (25th–75th percentile): 6.80 (6.20–9.63) µmol/L] and were defined as subjects with low fHcy. Participants had no clinical evidence of macroangiopathy (no history of ischemic heart disease, stroke, or peripheral macroangiopathy) and were taking no regular medication. Exclusion criteria for all groups were all the risk factors for atherosclerosis; use of antioxidant vitamin supplements and folate, hormone replacement therapy, or anti-inflammatory medication during the past year; existence of any inflammatory disease or cancer; and laboratory evidence of liver or hematologic abnormalities. Patients with coronary artery disease or peripheral arterial disease were excluded. All subjects had normal renal function (normal creatinine concentrations and no macroalbuminuria as defined by an albumin-to-creatinine ratio >300 µg/mg). Creatinine clearance as an estimate of the glomerular filtration rate [calculated by the Cockcroft-Gault formula (16)] was >90 mL/min in all subjects. All measurements were performed between 0800 and 1000 after the subjects had fasted for 12 h. Subjects rested in a supine position in a dark, quiet room maintained at a constant temperature of 22–25 °C for 30 min before measurement of forearm blood flow was measured and blood sampling was performed. Participants were initially allocated into 2 groups according to fHcy concentrations and were then randomly assigned (by using the dynamic random allocation method) into 2 equally sized groups that received either methionine (100 mg/kg body wt) plus 800 IU α-tocopherol and 2 g vitamin C (n = 15) or methionine (100 mg/kg body wt) with no additional antioxidant vitamins (placebo group), all diluted in 150 mL orange juice. Subjects remained in the examination room after they received the intervention, and the protocol was repeated 4 h after methionine loading (PML). The protocol was approved by the Institutional Ethics Committee, and informed consent was given by each subject.

Forearm blood flow measurements

Forearm blood flow was measured by using strain gauge plethysmography as we described previously (EC-400 and NIIVP3 software; D.E. Hokanson, Inc, Bellevue, WA; 17–19). Reactive hyperemia was expressed as the percentage change in forearm blood flow from baseline to the maximum flow during postischemic hyperemia (17, 18). The forearm vasodilatory response to nitroglycerin was expressed as the percentage change in forearm blood flow from baseline to the maximum flow after sublingual administration of 0.4 mg nitroglycerin. Reactive hyperemia is a marker of endothelial function in forearm resistance vessels, because it is almost identical to the vascular responses to acetylcholine (20) and is blocked by the nitric oxide synthase inhibitor L-NAME (20, 21).

Biochemical measurements

Venous blood samples were obtained at baseline and at 4 h PML. After centrifugation at 2000 × g at 4 °C for 15 min, plasma or serum was collected and stored at −80 °C until assayed. Routine chemical methods were used to determine serum lipid concentrations, glucose, and serum creatinine. Enzyme-linked immunosorbent assays were used to determine serum concentrations of TNF-α, interleukin 6 (IL-6), oxidized LDL (Merland AB, Uppsala, Sweden), and von Willebrand factor (vWF) Asserachrom vWF Kit, Diagnostica Stago, Asnières sur Seine,
France). Plasma total homocysteine was determined by fluorokinetic polarity immunoassay (AXSYM analyzer; Abbott Laboratories, Abbott Park, IL). ADMA concentrations were measured by enzyme-linked immunoassorbent assay (DLL Diagnostika GMBH, Hamburg, Germany). The sensitivity of this particular ADMA kit is 0.05 μmol/L, and its specificity is 100% for ADMA, <0.02% for arginine, 1.0% for NMMA, and 1.2% for symmetrical dimethylarginine (SDMA). In young healthy individuals, we found a range of values from 0.37 to 0.90 μmol/L [median (25th-75th percentile): 0.59 (0.53–0.685) μmol/L].

**Statistical analysis**

Continuous variables were tested for normal distribution by use of the Kolmogorov–Smirnov test. Data not normally distributed were log-transformed for analysis and are presented in the nonlogarithmic format as medians (25th-75th percentiles). Data normally distributed are presented as means ± SEMs. Baseline comparisons between the 4 groups were performed by one-way analysis of variance (ANOVA) for multiple comparisons followed by Bonferroni’s post hoc correction or the Kruskal-Wallis test as appropriate. When comparisons between 2 groups were performed, the Mann Whitney U test or unpaired t tests were used for nonnormally and normally distributed variables, respectively. The effect of methionine loading on each variable was examined by ANOVA for repeated measurements. The 2- and 3-factor interactions between time (before and after methionine loading), intervention (vitamins or placebo), and the presence of elevated baseline fHcy (high or low fHcy) were also examined by ANOVA for repeated measurements. Correlations between variables were assessed with Spearman’s correlation coefficient. A two-tailed P value < 0.05 was considered statistically significant. Analyses were performed by using the SPSS statistical package for WINDOWS, version 12.0 (SPSS Inc, Chicago, IL).

**RESULTS**

There were no significant differences between the study groups at baseline in any of the variables examined (Table 1). There was no significant correlation between creatinine clearance and fHcy (r = −0.136, P = 0.475), ADMA (r = 0.135, P = 0.476), or any inflammatory marker at baseline.

**Homocysteine and oxidized LDL**

Subjects with high fHcy had significantly higher concentrations of oxidized LDL than did those with low fHcy at baseline (P < 0.01; Table 2), and fHcy concentrations were strongly correlated with oxidized LDL concentrations (r = 0.600, P = 0.0001; Figure 1). Serum concentrations of oxidized LDL were significantly increased 4 h PML in the total placebo group (from 56.3 ± 8.2 to 107.6 ± 8.9 IU/L; P < 0.001), an effect blocked by administration of vitamins (from 61.6 ± 5.3 to 61.8 ± 9.7 IU/L; NS), whereas the change in the placebo group was significantly greater than the change in the vitamins group (P < 0.0001 for time × intervention interaction; Table 2). The same inhibitory effect of vitamins on the methionine loading-induced increase in oxidized LDL was observed both in subjects with high and in those with low fHcy concentrations at baseline (P = NS for time × baseline fHcy interaction; Table 2).

**Homocysteine and the vascular endothelium: effects on von Willebrand factor and reactive hyperemia**

Subjects with high baseline fHcy had higher vWF than did subjects with low fHcy (P < 0.05; Table 2). vWF was correlated with both fHcy (r = 0.506, P = 0.004; Figure 1) and oxidized LDL (r = 0.418, P = 0.021). Plasma vWF was similarly increased 4 h PML in both the total group of vitamin-treated subjects (from 59.2 ± 5.7 to 76.9 ± 6.2%; P < 0.01) and in the total placebo-treated group (from 59.6 ± 5.4% to 76.1 ± 4.9%; P < 0.01; NS for time × intervention interaction). Similarly, there was no significant effect of baseline fHcy concentrations on methionine-induced changes in vWF (P = NS for time × baseline fHcy interaction; Table 2).

Subjects with high fHcy had lower reactive hyperemia than did subjects with low fHcy (P < 0.05; Table 2), and reactive hyperemia was negatively correlated with fHcy concentrations (r = −0.487, P = 0.006). Methionine loading decreased reactive hyperemia in both the placebo (from 96.7 ± 9.6 to 52.2 ± 6.5% P < 0.001) and the vitamin-treated (from 86.6 ± 10.5 to 41.1 ± 6.2% P < 0.01) group similarly 4 h PML (P = NS for time × intervention interaction; Table 2). Maximum hyperemic forearm blood flow was similarly decreased 4 h PML in both the placebo (from 7.5 ± 0.5 to 5.7 ± 0.4 mL/100 mL tissue/min P < 0.05) and the vitamin-treated (from 8.4 ± 0.9 to 6.9 ± 0.7 mL/100 mL tissue/min) group (P = NS for time × intervention interaction). The forearm hyperemic response (as evaluated by the time-flow curves) was similarly decreased 4 h PML in both groups, independently of baseline fHcy concentrations (P < 0.05 for both versus baseline and P = NS for the time × intervention and time × baseline fHcy interactions; Figure 2).

**Homocysteine and the inflammatory process**

Subjects with low fHcy concentrations had lower IL-6 than did those with high fHcy concentrations (P < 0.05; Table 2), and fHcy was correlated with IL-6 (r = 0.380, P = 0.048). IL-6 was significantly increased 4 h PML in the placebo group (from 1.54 ± 0.19 to 1.96 ± 0.23 pg/mL; P < 0.01), an effect prevented by antioxidant vitamins (from 1.38 ± 0.20 to 1.40 ± 0.17; NS). The increase in IL-6 in the placebo group was significantly higher than the increase in the vitamin group (P < 0.05 for the time × intervention interaction). Baseline fHcy concentrations could not modify the response of the methionine-induced elevation in IL-6 to vitamin administration (P = NS for the time × baseline fHcy and time × intervention × baseline fHcy interactions; Table 2).

Although serum TNF-α concentrations were slightly higher in subjects with high fHcy, this difference was not significant. For TNF-α concentrations, there was no significant time × intervention or time × baseline fHcy interaction (Table 2).

**Homocysteine and ADMA**

Serum ADMA was not significantly different between subjects with low or high fHcy (Table 2). Similarly, fHcy was not correlated with ADMA concentrations at baseline (r = −0.060, P = 0.753; Figure 3). However, ADMA was significantly increased after methionine loading in the placebo-treated group (from 0.616 ± 0.040 to 0.817 ± 0.038 μmol/L; P < 0.01), an effect not prevented by antioxidants (from 0.589 ± 0.032 to 0.775 ± 0.048 μmol/L; P < 0.01; NS for time × intervention interaction). Baseline fHcy concentrations could
Effects of antioxidants on methionine-induced changes in endothelial function, inflammatory process, and markers of endothelial cell integrity

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<th>Vitamin group</th>
<th>Placebo group</th>
<th>Vitamin group</th>
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<td>Baseline¹</td>
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<td>66.1 ± 6.1</td>
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<td>vWF (%)</td>
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<td>51.0 ± 8.5</td>
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<td>9.8 ± 1.0</td>
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<td>RH (%)</td>
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<td>Baseline¹</td>
<td>85.1 ± 16.7</td>
<td>68.7 ± 7.6</td>
<td>106.9 ± 10.4</td>
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<td>TNF-α (pg/mL)</td>
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<td>ADMA (μmol/L)</td>
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<td>Baseline</td>
<td>0.58 ± 0.06</td>
<td>0.57 ± 0.05</td>
<td>0.65 ± 0.05</td>
<td>0.60 ± 0.02</td>
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<tr>
<td>4 h PML</td>
<td>0.79 ± 0.06³</td>
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<tr>
<td>Baseline</td>
<td>16.5 (13.4–46.3)³</td>
<td>24.9 (14.5–33.5)³</td>
<td>7.8 (6.2–10.3)³</td>
<td>6.6 (6.2–9.0)³</td>
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<td>4 h PML</td>
<td>45.3 (35.5–88.0)³</td>
<td>51.7 (34.8–63.5)³</td>
<td>26.2 (18.5–31.3)³</td>
<td>22.7 (18.1–30.8)³</td>
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</tbody>
</table>

¹ All values are ± SEM unless marked otherwise. ox-LDL, oxidized LDL; 4 h PML, 4 h after methionine loading; vWF, von Willebrand factor; FBF, forearm blood flow; RH, forearm vasodilatory response to reactive hyperemia; NTG, forearm vasodilatory response to nitrate; IL-6, interleukin 6; TNF-α, tumor necrosis factor α; ADMA, asymmetric dimethylarginine; fHcy, fasting homocysteine.
² Two-factor interactions between time (T), before or after methionine loading), intervention (I, vitamin or placebo), and the presence of elevated baseline fHcy (H, high or low) were evaluated by ANOVA for repeated measurements. There was no significant 3-factor interaction (T × I × H) for any of the examined variables.
³,⁴,⁵ P < 0.01 and P < 0.05, respectively, for comparisons of baseline values between subjects with low and those with high fHcy (one-way ANOVA for multiple comparisons or Kruskal-Wallis test as appropriate).
³ Significantly different from baseline, P < 0.05.
⁶ All such values are median (25th–75th percentile).

There was no correlation between the examined inflammatory markers or their changes during methionine loading and baseline ADMA or its methionine-induced changes (data not shown). Although ADMA was not correlated with reactive hyperemia at baseline (Figure 3), a significant correlation was observed after loading (Figure 3).

**DISCUSSION**

We have shown that high fHcy is associated with impaired endothelial function, higher concentrations of oxidized LDL,
and increased inflammatory status. Methionine-induced homocystinemia increased the expression of proinflammatory cytokines; elevated plasma concentrations of vWF, ADMA, and oxidized LDL; and impaired the forearm hyperaemic response. Although administration of high-dose antioxidants prevented the methionine-induced increase in oxidized LDL and IL-6, it failed to prevent the decrease in the forearm hyperaemic response and the increase in concentrations of vWF and ADMA.

Homocysteine, oxidative stress, and endothelial injury

The oxidative hypothesis seems to be the most well-established link between homocysteine and atherosclerosis, although it was recently questioned (7, 22, 23). Despite initial reports that antioxidants prevent the development of methionine-induced endothelial dysfunction (5, 24), other studies have shown that blocking the increase of oxidative stress does not necessarily prevent the methionine-induced impairment of endothelial function (25, 26). We have shown that both chronic and methionine-induced homocystinemia are associated with increased oxidized LDL, endothelial dysfunction, and higher circulating vWF, which is a surrogate marker of endothelial function known to be increased in homocystinemia (27, 28).

Although high-dose antioxidants prevented the methionine-induced increase in concentrations of oxidized LDL, they failed to prevent the impairment of endothelial function or the increase in vWF, which suggests that other mechanisms not regulated by free radicals may also play an important role in homocysteine-induced endothelial dysfunction.

Homocysteine and the inflammatory process

The cytokines IL-6 and TNF-α depress nitric oxide production (29) and enhance the release of vWF (30) from vascular endothelium. Free radicals trigger the release of proinflammatory cytokines (31), whereas oxidized LDL activates redox-sensitive pathways (such as nuclear factor-κB) regulating the expression of these cytokines (32, 33). Therefore, oxidized LDL may be a link between homocystinemia and increased inflammatory states (34, 35), although the underlying mechanisms are still unclear.

We have shown that subjects with high fHcy concentrations have higher concentrations of IL-6 than do subjects with low fHcy concentrations. Similarly, methionine-induced homocystinemia increased IL-6 concentrations at 4 h PML, an effect prevented by antioxidants, which suggests that free radicals induce the expression of proinflammatory cytokines. However, despite the blockade of the methionine-induced increase in oxidative stress and proinflammatory cytokines by antioxidants, endothelial function was not preserved, which suggests that mechanisms mediated by homocysteine itself or by pathways activated during the biochemical conversion of methionine to homocysteine may decrease nitric oxide bioavailability independently from the increase in oxidative stress.

Homocysteine and asymmetrical dimethylarginine
eNOS can be inhibited by naturally occurring analogues of the nitric oxide precursor L-arginine, such as ADMA (36). In vitro evidence suggests that homocysteinemia may affect ADMA release, partly as a result of impaired activity of DDAH, which is the enzyme responsible for ADMA metabolism (37). Oxidized LDL impairs DDAH activity and increases ADMA elaboration.

![Graph A](image1.png)

**FIGURE 1.** Fasting plasma homocysteine (fHcy) concentrations at baseline were correlated with serum concentrations of oxidized LDL (ox-LDL; n = 30) and plasma concentrations of von Willebrand factor (vWF; n = 30). Plasma concentrations of fHcy were not normally distributed and were log-transformed for univariate analysis.

**FIGURE 2.** Methionine loading induced a similar mean (±SEM) decrease in hyperemic forearm blood flow (FBF), which is a marker of endothelial function, in both the placebo (A; n = 15) and the antioxidant-vitamin-treated (B; n = 15) groups 4 h after methionine loading (P < 0.05 for both). Comparisons of hyperemic FBF before (●) and after (□) methionine loading were performed by using ANOVA for repeated measurements. There was no interaction between the effect of methionine loading and either treatment (vitamin or placebo) or fasting homocysteine concentrations (high or low).
by endothelial cells in homocystinemia (10), but at a clinical level, chronic antioxidant treatment with vitamin E fails to lower ADMA in patients with chronic renal disease (38). On the other hand, the conversion of methionine to homocysteine drives the formation of ADMA through a respective increase in \(N\)-methyltransferase activity (39). Arginine residues in proteins are methylated by protein arginine methyltransferases (PRMTs), and proteolysis of these proteins results in ADMA formation (36, 39). Although ADMA is eliminated from the body by renal excretion (40), recent studies suggest that only \(\approx20\%\) of ADMA is eliminated by renal excretion. In patients with primary renal disease, ADMA is already elevated, whereas the glomerular filtration rate is still normal, and the correlation between ADMA and renal function is rather weak (41). Finally, it has been proposed that ADMA synthesis is also triggered by proinflammatory cytokines (10, 42), although long-term exposure to proinflammatory cytokines may decrease ADMA by up-regulating DDAH (14). Evidence suggests that chronic infection by cytomegalovirus (43) decreases the activity of DDAH, an effect also observed in rats treated with endotoxin to induce iNOS (44), which suggests that DDAH activity is largely regulated by inflammatory stimuli.

In vivo, methionine-induced homocystinemia is accompanied by an elevation of ADMA concentrations (8, 40), but the role of ADMA in chronic homocystinemia is controversial (45). Homocysteine-lowering treatment with oral combined B vitamins for 8 wk failed to improve endothelium-dependent vasodilation in patients with peripheral arterial disease and hyperhomocysteinemia, whereas L-arginine improved endothelial function in these patients (46). In contrast, in other studies it was shown that folate treatment can reduce ADMA in patients with hyperhomocysteinemia (47). These contradictory results imply that the widely used model of methionine-induced homocystinemia may not correspond fully to clinical homocystinemia, because the increase in homocysteine concentrations is due to over-activation of the pathway converting methionine to homocysteine, which is a biochemical concept not observed in chronic homocystinemia.

We found that methionine loading acutely increased oxidative stress, proinflammatory cytokines, and ADMA concentrations. Although high-dose antioxidant treatment blocked the increase in oxidized LDL and proinflammatory cytokines, however, it failed to prevent the increase in ADMA and the impairment in

![Graph 1](https://example.com/graph1.png)

![Graph 2](https://example.com/graph2.png)

**FIGURE 3.** No correlation was found between fasting homocysteine (fHcy) concentrations and asymmetrical dimethylarginine (ADMA) concentrations at baseline (\(n = 30\)). Although ADMA concentrations were not significantly correlated with reactive hyperemia at baseline (RH; \(n = 30\)), a significant correlation was observed 4 h after methionine loading (4 h PML; \(n = 30\)). Plasma concentrations of fHcy were not normally distributed and were log-transformed for univariate analysis.
endothelial function. These findings suggest that the rapid increase in ADMA is partly responsible for the endothelial dysfunction in acute homocysteinemia and that its synthesis is not mediated by free radicals or proinflammatory cytokines. However, high fHcy was not associated with higher ADMA, which implies that ADMA may be largely increased in experimental models by using methionine to induce homocysteinemia, possibly as a result of the transmethylation of arginine (9, 39). The role of ADMA in chronic homocysteinemia needs further evaluation. In chronic homocysteinemia, other regulatory mechanisms (such as the long-term down-regulation of PRMTs or the up-regulation of DDH gene expression) may prevent the increase in ADMA concentrations (14). Finally, in contrast with previous reports of a correlation between renal function and fHcy concentrations (48), the lack of any association between creatinine clearance and fHcy in our population of young healthy individuals suggests that the contribution of a possible subclinical renal impairment could not account for the observed variations in baseline fHcy in the study population.

Study limitations
A limitation of the study was the use of orange juice (150 mL) to dilute the methionine and antioxidant vitamins, which contains 50 mg vitamin C (49), which may have had some effect on the overall response to methionine loading in both groups. Furthermore, the study was not designed to address the hypothesis that vitamin C and E (vitamin C (49), which may have had some effect on the overall response to methionine loading in both groups. Furthermore, the study was not designed to address the hypothesis that vitamin C and α-tocopherol without methionine loading have any effect on concentrations of IL-6 and TNF-α in healthy individuals.

Conclusions
We have shown that the increase in oxidative stress observed during methionine-induced homocysteinemia stimulates the release of proinflammatory cytokines, and that this effect is prevented by high-dose antioxidant treatment. Although antioxidants prevent the increase in oxidized LDL and proinflammatory cytokines, however, they fail to prevent the development of endothelial dysfunction and the elevation of ADMA. This is one of the first studies (50) showing a biological effect of ADMA in an acute setting in humans and provides further insights into the mechanisms regulating its release in human homocysteinemia. However, ADMA is not elevated in subjects with high fasting homocysteine concentrations, and its role in chronic homocysteinemia requires further evaluation.

CA and DT designed the study, analyzed the data, and prepared the manuscript. CA, KM, and GB performed the clinical part of the study. CV and CS supervised the study and contributed to the writing of the manuscript. None of the authors had a conflict of interest.

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