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Plasma cysteine and glutathione are independent markers of postmethionine load endothelial dysfunction

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Abstract

Objectives: Oxidative stress caused by acute hyperhomocysteinemia impairs endothelial function in human arteries. We sought to identify markers of endothelial dysfunction during methionine-induced hyperhomocysteinemia.

Design and methods: 35 subjects underwent flow-mediated dilation (FMD) of the brachial artery by high-resolution ultrasonography and fasting blood samples before and 3 h postmethionine load (PML). Clinical, conventional biochemical, and redox status (plasma total and reduced homocysteine, glutathione, cysteine, cysteinylglycine, ascorbic acid, α-tocopherol, free malondialdehyde, blood glutathione) data were sequentially entered into an univariate and multivariate stepwise linear regression analysis to evaluate their relation with the dependent variable FMD.

Results: Median [interquartile range] FMD decreased from 4.1% [2.8–6.3] to 3.2% [0.7–4.3] PML ($P=0.02$). At the multivariate analysis PML total cysteine ($\beta=-0.008$, $P=0.002$) and glutathione ($\beta=0.21$, $P=0.005$) were the only independent variables associated with FMD after methionine, adjusted for baseline FMD.

Conclusions: Elevated plasma total cysteine and decreased plasma total glutathione levels were associated with abnormal FMD PML. Cysteine and glutathione are stronger markers of endothelial dysfunction than clinical and all other biochemical variables explored.

Keywords: Endothelial function; Homocysteine; Flow-mediated vasodilation; Antioxidants

Introduction

Endothelial dysfunction is a key factor in the development of atherosclerosis and thrombosis [1]. In patients with chronic hyperhomocysteinemia, endothelium-dependent dilation is impaired [2] and this metabolic abnormality may trigger the initiation and progression of atherosclerosis and/or thrombosis. Flow-mediated dilation (FMD) can be acutely impaired by transient elevations of plasma homocysteine induced by oral methionine [3–6], providing a model to assess the mechanisms underlying endothelial dysfunction. The implicated candidates of homocysteine-induced endothelial dysfunction include a decrease in nitric oxide bioavailability [2] due to either a direct impairment of its synthesis [7] or mediated by promotion of oxidative damage [8,9]. However, previous studies in healthy subjects [10,11] do not support oxidative stress as mechanism of postmethionine load (PML) endothelial dysfunction and other studies did not find changes in endothelial function following methionine test [12]. Changes in aminothiol status during transient hyperhomocysteinemia [13] may influence the vasomotor response. Conversion of homocysteine back to methionine (remethylation) and transsulfuration to cysteine are the major metabolic pathways to reduce homocysteine concentrations in cells and in blood. PML,

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homocysteine transsulfuration is favored instead of remethylation through activation of cystathionine β-synthase, with an increased homocysteine to cysteine conversion. Glutathione (GSH), in turn, synthesized intracellularly by cysteine, glutamate and glycine, plays a crucial role in the antioxidant cell protection and constitutes the major intracellular non-protein sulphydryl pool, rather important intermediate in the action of nitric oxide [14]. Thus, the dynamics of the other thiols, such as cysteine and GSH, may influence vascular reactivity during acute hyperhomocysteinemia.

In order to assess the influence of plasma redox thiol status on the endothelial function during acute hyperhomocysteinemia in humans, we simultaneously evaluated FMD by brachial ultrasonography and biochemical markers of circulating redox status in 35 subjects.

Methods

Study population

A group of 35 consecutive subjects (22 males, 45 [31–54] years) was referred to our Center for the evaluation of endothelial function because of presence of risk factors for atherosclerosis, including hyperhomocysteinemia; all of them underwent FMD and redox status measurements before and after a methionine loading. Clinical, biochemical and genetic characteristics of the study population are presented in Table 1. Cigarette smokers abstained from smoking on the day of the study; medications were withdrawn at least 72 h before the study and all subjects were not allowed to take vitamin supplements for 3 weeks before. None had history of immunological, neoplastic diseases or renal function impairment. Hypertension was defined as systolic blood pressure>140 mm Hg and/or diastolic blood pressure>90 mm Hg on repeated measurements or chronic use of antihypertensive drugs [15]; hypercholesterolemia as total cholesterol level>240 mg/dL and/or LDL cholesterol level>160 mg/dL or the need for lipid-lowering medication [16]; diabetes mellitus as fasting glucose levels ≥126 mg/dL or the need for insulin or oral hypoglycemic agents [17]. All participants gave informed, written consent and the study protocol was approved by the Niguarda Ca' Granda Hospital ethics committee.

Table 1
Clinical, biochemical and genetic characteristics in the 35 subjects

	All cases (n=35)
Age, years	45 [31–54]
Male gender, *n* (%)	22 (63%)
BMI, kg/m^2	26 [22–30]
Smokers, *n* (%)	11 (31%)
Diabetes, *n* (%)	0 (0%)
Hypertension, *n* (%)	12 (34%)
Hypercholesterolemia, *n* (%)	9 (26%)
Hyperhomocysteinemia, *n* (%)	19 (54%)
ACE inhibitors, *n* (%)	5 (14%)
β-Blockers, *n* (%)	2 (6%)
Calcium-channel blockers, *n* (%)	5 (14%)
Statins, *n* (%)	4 (11%)
Antiplatelet agents, *n* (%)	9 (26%)
Fasting glucose, mg/dL	95 [88–103]
Total cholesterol, mg/dL	188 [153–217]
HDL cholesterol, mg/dL	50 [43–61]
LDL cholesterol, mg/dL	113 [90–148]
Triglycerides, mg/dL	97 [59–130]
Creatinine, mg/dL	0.88 [0.75–0.96]
Serum folate, ng/mL	3.9 [3.0–6.2]
Vitamin B_{12}, pg/mL	363 [259–469]
γ-Glutamyltransferase, U/L	25 [12–45]
MTHFR genotype, *n* (%)	
Wild type	12 (34%)
Homozygote	12 (34%)
Heterozygote	11 (32%)

Data are expressed as number of patients (percentage) and median value [interquartile range].
BMI, body mass index; ACE, angiotensin-converting enzyme; MTHFR, methylenetetrahydrofolate reductase.

Study protocol

After an overnight fast, venous blood samples were obtained in resting, supine condition in order to assess reduced and total plasma aminothiols (homocysteine, cysteine, GSH, and cysteinylglycine), blood GSH, ascorbic acid and α-tocopherol, free malondialdehyde as index of lipid peroxidation, vitamin B_{12}, serum folate, glucose, creatinine, γ-glutamyltransferase, total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides. An antecubital vein of the dominant arm was cannulated for blood sampling. FMD and nitrate-related dilation of the brachial artery were assessed before and 3 h after oral methionine (L-methionine 3.8 g/m^2 body surface area). At 3 h PML, blood samples were drawn to measure concentration of aminothiols, ascorbic acid, α-tocopherol, and malondialdehyde. During the study only water was allowed.

Arterial reactivity studies

The non-invasive determination of endothelium-dependent and -independent dilation was performed according to Celermajer et al. [18,19] with modifications for continuous measurements proposed by Stadler et al. [20], and according to the guidelines for the ultrasound assessment of endothelial-dependent FMD of the brachial artery [21].

The brachial artery (2–15 cm above the elbow) was scanned in longitudinal section by a B-mode ultrasound device equipped with a 7.5–10.0 MHz linear array transducer (AU4, ESAOTE Biomedica). Depth and gain settings were appropriately selected in order to optimize images of the lumen/arterial wall interface. Machine operating parameters were not changed during the study. All experiments were performed in a temperature-controlled room (24±2°C). The subjects laid at rest for at least 15 min before each scan was recorded. Scans were taken at rest, during reactive hyperemia, again at rest, and after sublingual glyceryltrinitrate in order to evaluate the endothelium-independent dilation.

For the FMD measurements, the diameter of the brachial artery was recorded: (a) for 300 s at rest, (b) during the 5 min of ischemia obtained by inflating a pneumatic tourniquet placed on the forearm to a pressure 50 mm Hg above the individual systolic blood pressure and (c) for 3 min after the pneumatic tourniquet deflation, i.e., the reactive hyperemic phase. Measurements of

brachial arterial diameter were performed with 5-s interval. Brachial FMD was determined as the percentage change from baseline to the point of maximum dilation after release from ischemia. Peak dilation was observed, in average, 56 s after cuff deflation. Diameter alteration returned to normal within 2–5 min post-ischemia.

After 15 min of rest, to allow vessel recovery, a further baseline scan was obtained. Glyceryltrinitrate (300 μg) was administered and brachial artery diameter was continuously recorded for at least 15 min. All the above described procedures were repeated after 3 h PML. Brachial artery diameter was measured as the distance between the near and far media/adventitia interfaces measured during the end-diastolic phase of each cardiac cycle. Vessel diameter was measured by an expert observer (L.P.), the same investigator who collected the images, unaware of the clinical details and of the stage of the experiment, by using a specific software. The time course of post-ischemic or post-nitrate diameter changes were visualized by plotting the diameter measurements (each diameter value is the average of 3 measurements) versus time. To assess the intra-variability of the technique, brachial scans from 20 men (mean age 58 ± 9.5 years) were carried out, on two separate occasions, by the same observer at a mean interval of 0.8 ± 1.2 (range 0.11–4.9) weeks. In this reproducibility study a mean difference of 1.3% in FMD over time was obtained (on a baseline vasodilatation of about 5%). This figure compared favorably with data provided by other authors [18,19]. Blood pressure and heart rate were continuously recorded from the middle finger of the dominant arm (2300 Finapress, Ohmeda).

Chemical analyses

Samples were prepared and analyzed for thiols and vitamins immediately after collection. Plasma reduced and total forms of homocysteine and other thiols, blood reduced GSH (index of GSH concentrations in circulating cells) were determined according to methods validated in our laboratory [22,23]. Thiol separation was performed by high-performance liquid chromatography (ProStar, Varian, Surrey, UK), as previously described [22]. Intra- and interassay precision, determined as coefficient of variation, ranged between 0.60% and 6.14% and between 2.10% and 7%, respectively. Hyperhomocysteinemia (>12 μmol/L) was defined according to data obtained in our laboratory from a large population of healthy subjects with a wide age range, without risk factors or vascular events, using the 90th percentile of control distribution, and was similar to those reported in the literature [24]. Malondialdehyde levels were determined in −80°C stored plasma by a reference method based on gas chromatography-mass spectrometry technique with isotope dilution [25]. Plasma ascorbic acid and α-tocopherol levels were analyzed by isocratic high-performance liquid chromatography separation, as previously described [26]. α-Tocopherol values were normalized for the corresponding total cholesterol values. Vitamin B_{12} and folate were measured by competitive immunoassay using direct chemiluminescence, while glucose, creatinine, γ-glutamyltransferase, total cholesterol and triglycerides were determined using standard laboratory methods. HDL cholesterol was measured after precipitation with dextran sulphate–magnesium and LDL cholesterol was calculated using the Friedewald's method.

Genetic analysis

DNA was extracted from aliquots of blood cellular fraction and stored at −80°C. The methylenetetrahydrofolate reductase 677C→T genotype was identified by PCR amplification, followed by HinfI restriction digestion, while cystathionine β-synthase 833T→C genotype was identified by PCR amplification, followed by BsrI restriction digestion. Digestion products were separated by 3% agarose gel electrophoresis, stained with ethidium bromide. The methylenetetrahydrofolate reductase wild type allele gave a 198-bp fragment, and insertion variant gave two fragments of 175 bp and 23 bp. The cystathionine β-synthase wild type allele gave 174-bp fragment, and insertion variant gave two fragments of 132 bp and 42 bp [27].

Statistical analysis

Results are expressed as median and interquartile ranges [I–III]. Baseline and PML measurement differences were assessed by paired Student's *t* test for continuous variables or Wilcoxon signed rank test for non-normally distributed variables. Univariate and multivariate stepwise linear regression analysis was applied to evaluate the relation between the dependent variable FMD PML, both adjusted and not adjusted for baseline FMD, and the other independent clinical, conventional biochemical, and redox status variables. The statistical analyses were carried out with the Statistical Package for the Social Sciences (SPSS) release 10.0 for Windows and S-Plus release 6.0. Two-sided $P<0.05$ values were considered statistically significant.

Results

Clinical, conventional biochemical and genetic characteristics in the overall population are presented in Table 1. No mutation was found for cystathionine β-synthase.

Median plasma total homocysteine was 14.3 [8.3–24] μmol/L at baseline and increased to 28 [19–39] μmol/l ($P<0.001$) PML (Table 2). Plasma reduced homocysteine, cysteine, and cysteinylglycine, as well as blood reduced GSH significantly increased PML ($P<0.001$ for all variables). Malondialdehyde concentration was 0.40 [0.30–1.06] μmol/L and did not change after methionine (0.44 [0.31–0.91] μmol/L, $P=0.76$).

Median FMD decreased from 4.1% [2.8–6.3] to 3.2% [0.7–4.3] PML ($P=0.02$). The dilation to sublingual glyceryltrinitrate was 21% [18–25] at baseline and 19% [16–23] PML ($P=0.06$). Individual changes in FMD and dilation to glyceryltrinitrate from baseline to PML are depicted in Fig. 1. Baseline FMD was not correlated to any clinical, biochemical and redox status variables. At univariate analysis PML FMD, adjusted by baseline FMD, was significantly and negatively correlated to age ($P=0.0001$), hypertension ($P=0.016$), hypercholesterolemia ($P=0.02$), fasting glucose ($P=0.03$),

Table 2
Baseline and PML plasma thiols, vitamins, blood GSH, MDA, FMD and dilation to sublingual glyceryltrinitrate

	Baseline	PML	*P*
Total thiols			
Hcy	14.3 [8.3–24]	28.0 [19–39]	0.0001
Cysteinylglycine	22 [19–24]	22 [18–27]	0.93
GSH	5.5 [3.6–6.7]	5.0 [3.8–7.0]	0.59
Cysteine	236 [184–301]	246 [175–293]	0.27
Reduced thiols			
Hcy	0.25 [0.15–0.84]	0.83 [0.54–1.53]	0.0001
Cysteinylglycine	2.4 [2.1–3.3]	2.9 [2.5–3.9]	0.0001
GSH	1.6 [1.0–2.1]	1.5 [1.0–2.1]	0.26
Cysteine	8.5 [6.4–9.4]	10.9 [9.1–12.2]	0.001
Ascorbic acid	46 [34–57]	44 [32–58]	0.99
α-Tocopherol	0.13 [0.11–0.18]	0.15 [0.11–0.17]	0.36
MDA	0.40 [0.30–1.06]	0.44 [0.31–0.90]	0.76
Blood GSH	380 [224–541]	533 [418–716]	0.0001
FMD (%)	4.1 [2.8-6.3]	3.2 [0.7-4.3]	0.02
GTN (%)	21 [18-25]	19 [16-23]	0.06

Data are expressed as median value [interquartile range]. Biochemical measurements are expressed in μmol/L. α-Tocopherol was normalized for total cholesterol.
PML, postmethionine loading; Hcy, homocysteine; GSH, glutathione; MDA, free malondialdehyde; FMD, flow-mediated dilation; GTN, dilation to sublingual glyceryltrinitrate.

total cholesterol ($P=0.004$), LDL cholesterol ($P=0.01$), triglycerides ($P=0.007$), γ-glutamyltransferase ($P=0.01$), basal and PML total cysteine ($P=0.02$ and $P=0.002$, respectively), while it was significantly and positively correlated to basal and PML total GSH ($P=0.01$ and $P=0.005$, respectively). In the multivariate analysis, only PML total cysteine ($\beta=-0.008$, $P=0.002$) and total GSH ($\beta=0.21$, $P=0.005$) were independent variables associated with PML FMD, adjusted by baseline FMD (Fig. 2). Even not adjusting univariate and multivariate analysis by baseline FMD, PML total cysteine ($\beta=-0.008$, $P=0.004$) and total GSH ($\beta=0.26$, $P=0.004$) remained independently associated with PML FMD.

In our study population we did not observe an inverse linear relationship between plasma total homocysteine and FMD, neither at baseline nor PML.

Discussion

The present study examined the relation between biochemical markers of redox status and endothelial function following methionine loading. The major observation is that in a group of subjects with risk factors for atherosclerosis the endothelial function after methionine is negatively correlated with plasma total cysteine levels and positively with plasma total GSH. Thus, in this study, to our knowledge for the first time, plasma cysteine is also an independent marker of endothelial dysfunction PML, after controlling for age, atherosclerotic risk factors, and potentially confounding covariates. Furthermore, in the overall population we found a significant increase in blood reduced GSH and plasma reduced cysteinylglycine after methionine. The increase of GSH concentrations in circulating cells confirms a successful buffer of free radical formation induced by the pro-oxidant stimulus, such as methionine loading. In fact, intracellular GSH seems to be reconstituted and to break down continuously into its by-product, as suggested by the rise of plasma cysteinylglycine levels. Our observations do not support any acute impairment of antioxidant defence following methionine loading and add further evidences showing that oxidative stress is not the mechanism responsible for endothelial dysfunction observed in acute hyperhomocysteinemia [11].

In our study population we did not observe the inverse linear relationship between plasma total homocysteine and FMD reported by others, either in hyperhomocysteinemic or in normal healthy subjects [3–5,28]. PML FMD, both adjusted and not adjusted for baseline FMD, appears to be dependent on changes in the redox state (cysteine and GSH) following methionine challenge, rather than on the extent of homocysteine increases.

Cysteine is a sulphydryl-containing amino acid which has recently attracted interest as potential risk factor for vascular disease [29–31]; like other amino thiols, cysteine may exist in plasma in reduced, oxidized, and protein-bound forms. The extensive and complex changes in cysteine and the overall aminothiols occurring in plasma and within the cells during acute hyperhomocysteinemia may modulate positively or negatively the FMD response to sudden increases in plasma homocysteine. Our findings indicate that circulating cysteine levels play an independent role on endothelial function following methionine challenge in middle-aged subjects. Our data also show that oxidative stress did not increase from

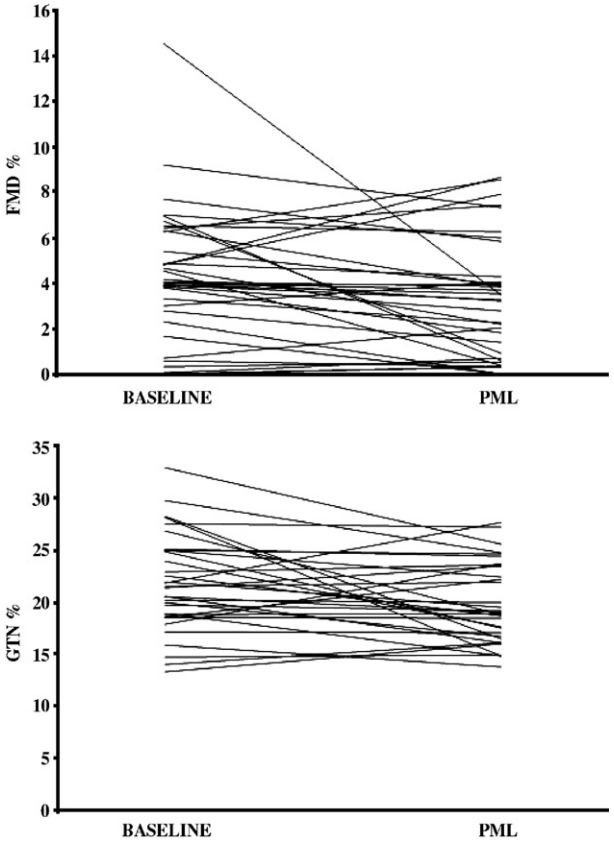

Fig. 1. Graph of individual changes in flow-mediated vasodilation (FMD%, upper panel) and glyceryltrinitrate dilation (GTN%, lower panel) from baseline to postmethionine loading (PML).

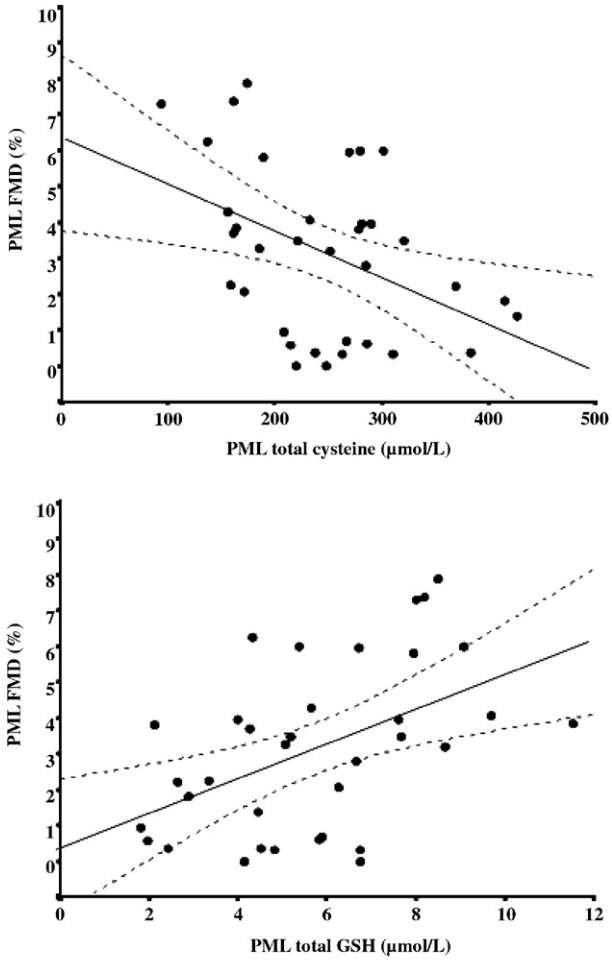

Fig. 2. The relation between PML total cysteine (upper panel) and total GSH (lower panel) and PML FMD, adjusted by baseline FMD, in the 35 subjects. The broken lines represent the 95% confidence interval of mean regression line.

baseline to 3 h PML, considering malondialdehyde as an index of oxidative status. The inconsistency in lipid peroxidation changes following acute hyperhomocysteinemia is in line with previous studies in human subjects [10–12] and suggests that the association between cysteine and endothelial dysfunction may be either the consequence of an altered equilibrium among aminothiols or the result of nitric oxide degradation, as an adduct with cysteine [32].

Sulphydryl-containing compounds, like cysteine and GSH, are required for activation of soluble guanylate cyclase by biotransformation to vasoactive nitric oxide [33]. Experimental studies have shown that increased intracellular GSH levels may augment the hemodynamic effect of organic nitrates [34]. Interaction between cysteine, GSH, nitric oxide, and nitric oxide donors may explain different behaviors of FMD and dilation to glyceryltrinitrate during acute hyperhomocysteinemia, independently of fasting and PML homocysteine values.

Study limitations

The major limitation is the quite small sample size, but we are in line with the most cited previous studies [2,3,10]. We did not perform the statistical analysis taking into account the delta-variables, because of the continuous changes in the thiol dynamics which does not allow catching of the effect of the methionine loading on the thiol reduced forms. We rather investigated whether more stable markers of thiol metabolism were significantly and independently correlated with PML endothelial dysfunction.

Conclusions

An increased concentration of plasma total cysteine and reduced concentration of plasma total GSH after methionine test are associated with abnormal FMD, both adjusted and not adjusted for baseline FMD, yielding incremental diagnostic value over clinical, biochemical, and the other explored redox variables, including baseline and PML homocysteine. The abnormal vascular response is not associated with increased oxidative stress, but rather with an altered aminothiol status following methionine loading. A single run determination of plasma aminothiols by high-performance liquid chromatography provides a cost-saving assessment of redox status before and after the methionine challenge.

The prognostic value of an altered plasma thiol equilibrium in predicting future cardiovascular events in subjects with risk factors has not been assessed yet. The pathophysiological implication of these findings in the progression of vascular dysfunction needs to be further investigated.

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