Peroxidation Indices and Total Antioxidant Capacity in Plasma During Hyperhomocysteinemia Induced by Methionine Oral Loading

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Hyperhomocysteinemia is a risk factor for vascular disease, although its mechanism of action is not fully clear. Different experimental studies have suggested that homocysteine (Hcy) exerts a pro-oxidant effect in the presence of metal ions (Fe and Cu). To test for a similar effect in vivo, we studied plasma markers of lipid and protein oxidation during hyperhomocysteinemia induced by an oral methionine load. Twenty-nine subjects (aged 61 ± 25 years; 17 women), 25 of whom underwent oral methionine (100 mg/kg) loading, were studied; in every case, we measured total plasma Hcy, malondialdehyde (MDA), conjugated dienes (DIE), and oxidized protein ([PTOX] carbonylic groups) in basal conditions and 4, 6, 8, and 24 hours after methionine loading. Four participants acted as controls. In every case, we also measured total plasma antioxidant capacity (ANTOX) in basal conditions and 8 hours after methionine loading. Eight hours after methionine loading, plasma Hcy increased from 17.6 ± 11.4 to 54.3 ± 31.6 nmol/mL, PTOX from 0.33 ± 0.18 to 0.71 ± 0.33 nmol/mg protein, DIE from 493 ± 163 to 590 ± 202 optical density units, and MDA from 1.66 ± 0.81 to 2.1 ± 0.93 nmol/mL. There was a significant correlation (Spearman's *r*) between Hcy and both PTOX ($r = .86$, $P = .01$) and MDA ($r = .47$, $P < .05$) 8 hours after methionine loading. No significant modifications of the plasma parameters were found during the observation period in controls. ANTOX at 8 hours was significantly (paired *t* test) reduced in probands (from 1.74 ± 0.59 to 1.14 ± 0.55 mmol/mL, $P = .014$); no significant difference was observed for plasma ANTOX in controls. Hyperhomocysteinemia due to oral methionine loading induced an increase in plasma oxidation markers. In the absence of hyperhomocysteinemia, no significant modifications were observed. These findings, together with the decrease in ANTOX and the corresponding increase in total plasma Hcy, are consistent with a pro-oxidant effect of acute hyperhomocysteinemia in vivo.

IT IS WELL KNOWN that patients with various congenital anomalies in homocysteine (Hcy) metabolism that cause extremely high levels of Hcy in the blood (>200 nmol/mL) and urine develop premature atherosclerotic and/or thromboembolic disease.[1] Clinical and epidemiological evidence[2-5] suggests that even a moderately elevated level of plasma Hcy (>15 nmol/mL), so-called hyperhomocysteinemia, is associated with vascular disease. Hyperhomocysteinemia is thus seen as an independent risk factor for vascular disease[6] affecting the coronary, cerebral, and peripheral vessels.[4-5,7-8] Indeed, it has been estimated that a 5-nmol/mL increase in plasma Hcy increases the risk of coronary heart disease to a similar extent as an increase in serum cholesterol of 0.5 mmol/L.[9]

The mechanism by which Hcy exerts its effect has not been clearly defined, although it is generally accepted that the accumulation of Hcy in plasma results in damage to the endothelial cells.[10,11] It has been suggested that oxidative damage may be involved. Hcy tends to undergo auto-oxidation when added to solution or to plasma, leading to the formation of homocystine, homocysteine-mixed disulfides, and homocysteine thiolactone. During these reactions, the reactive oxygen derivative H_2O_2 is generated and may be responsible for the cellular toxicity of Hcy.[12,13] However, some studies in vitro do not fully support the "oxidative" hypothesis,[14,15] although they all agree as to a possible pro-oxidative in vitro effect of Hcy in the presence of transition metal ions (Fe^{3+} and Cu^{2+}).

The aim of the present study is to assess the effect of acutely induced hyperhomocysteinemia in vivo on the oxidative plasma status. To this end, we analyzed oxidized protein and lipid and total plasma antioxidant capacity (ANTOX) in controls and in subjects exposed to acute hyperhomocysteinemia induced by an oral methionine load.

SUBJECTS AND METHODS

Twenty-nine subjects (mean age, 61 ± 25 years; 17 women) were studied. None of the subjects exhibited any significant kidney, liver, or endocrine dysfunction, nor was there any clinical or laboratory evidence of current inflammatory disease. They were included in the study regardless of prior basal Hcy levels, with prior provision of informed consent. Twenty-five underwent oral methionine loading with 100 mg/kg in 200 mL tea (probands), and 4 received 200 mL tea only (controls). The tea was tested to ensure that it was Hcy-free. In all subjects, we obtained plasma samples at baseline and 4, 6, 8, and 24 hours after oral methionine loading, or in controls, after consumption of the tea. In all samples, we measured plasma levels of Hcy, malondialdehyde (MDA), and conjugated dienes (DIE) (as indicators of plasma lipoperoxidation) and protein carbonyl groups (as indicators of oxidatively modified plasma proteins [PTOX]). Total plasma Hcy was

Table 1. Clinical Characteristics of the Subjects (N = 29)

Characteristic	Mean ± SD
Age (yr)	61 ± 25
Body mass index (kg/m²)	24.7 ± 1.1
Albumin (g/dL)	3.9 ± 0.3
Total plasma protein (g/dL)	6.9 ± 0.4
Hemoglobin (g/dL)	14.5 ± 1.5
Lymphocyte count (per μL)	2,336 ± 734
Protein catabolize rate (mg/dL)	0.51 ± 0.5
ESR (mm/h)	12.6 ± 3.1
Total cholesterol (mg/dL)	217 ± 24
Triglycerides (mg/dL)	191 ± 120
Creatinine clearance (mL/min)	79.2 ± 18.7

Abbreviation: ESR, erythrocyte sedimentation rate.

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Table 2. Plasma Parameters in Patients (n = 25) Subjected to Methionine Oral Loading (mean ± SD)

Parameter	Basal	4 h	6 h	8 h	24 h	χ^2 (4, 25)
Hcy (nmol/mL)	17.6 ± 11.4	44.3 ± 22.6	52.4 ± 29.3	54.3 ± 31.6	35.6 ± 28.3	77.347*
PTOX (nmol/mg protein)	0.33 ± 0.18	0.48 ± 0.26	0.62 ± 0.37	0.71 ± 0.33	0.57 ± 0.40	35.806*
DIE (optic density)	493 ± 163	562 ± 181	566 ± 233	590 ± 202	545 ± 182	19.953*
MDA (nmol/mL)	1.66 ± 0.81	1.91 ± 0.94	1.96 ± 1.31	2.10 ± 0.93	1.95 ± 0.99	32.032*

NOTE. χ^2 vaules are from the Friedman test of repeated measures.
*$P < .01$.

determined by high-performance liquid chromatography with fluorescence detection according to the method of Cornwell et al,[16] using cysteamine as an internal standard for a more accurate quantitative determination. Plasma MDA and DIE were assayed by spectrophotometric methods.[17,18] Protein oxidation, measured as protein carbonyl groups, was determined by spectrophotometric assay.[19]

We also measured ANTOX at baseline and 8 hours (ie, maximum plasma Hcy peak) after methionine or tea. ANTOX was determined by spectrophotometric assay[20] using a commercial kit (Randox Laboratories, Ardmore, UK).

Statistical analysis was performed with the SPSS package (SPSS, Chicago, IL). We used a paired Student's *t* test for intragroup comparisons of ANTOX before and 8 hours after methionine, and Friedman's test for intragroup statistical assessment of the variation in each parameter during methionine loading. Spearman's *r* correlation coefficient was used to determine correlations.

RESULTS

Clinical and biochemical data for the study participants are reported in Table 1. Tables 2 and 3 summarize the plasma levels (mean ± SD) of total Hcy and oxidative markers in the experimental and control groups, respectively. These tables also show the results of Friedman's test to assess the respective statistical relevance of alterations in the parameters over the test period. Figure 1 shows that ANTOX is significantly reduced (paired Student's *t* test, $P = .014$) 8 hours after oral methionine intake, whereas in the control group (subjects not exposed to acute hyperhomocysteinemia from oral methionine load), no significant variations of ANTOX levels are observed.

Figures 2 and 3 show the correlations between total plasma Hcy levels and protein oxidation (carbonylic groups, $r = .86$, $P < .01$) and lipid oxidation (MDA, $r = .47$, $P < .05$) indices 8 hours after an oral methionine load (when Hcy reaches the maximum plasma concentration), respectively. No significant correlations were observed between basal plasma Hcy and markers of protein oxidation ($r = .36$, $P = .061$) and lipid oxidation ($r = .31$, $P = .066$).

Figure 4 shows that in the experimental group, Hcy-induced protein oxidation 8 hours after methionine loading is inversely and significantly influenced by basal ANTOX ($r = -.79$, $P < .01$). These data remain statistically significant ($r = -.41$, $P < .05$) even if corrected for Hcy 8-hour plasma levels.

DISCUSSION

Our data demonstrate a statistically significant (Friedman's test) increase in plasma protein and lipid oxidation markers following an increase in plasma Hcy levels induced in vivo by oral methionine loading, and are consistent with a possible in vivo pro-oxidant effect of hyperhomocysteinemia. The slight alterations (no statistical significance by Friedman's test) in plasma oxidation indices in subjects not exposed to methionine loading (or, by inference, to hyperhomocysteinemia) and the significantly higher consumption of plasma ANTOX in the subjects exposed to hyperhomocysteinemia versus the controls are both findings that further support the putative "oxidative" capacity of Hcy.

The possible role of the orally induced "hypermethioninemia" in the observed oxidative alterations has not been directly evaluated. Nevertheless, it probably has poor relevance; during a methionine oral load, peak methionine plasma levels are reached 2 to 3 hours after intake, without the timing corresponding to the peak of molecular oxidative damage that we observed 8 hours after the intake (and in correspondence with the Hcy plasma peak). Moreover, our data in vitro (Ventura, unpublished data, March 1998) do not show a significant oxidative power for methionine at levels similar to those reached in our experimental model.

The oxidative effect of Hcy (Table 2) seems to involve both lipids (MDA and DIE) and protein (carbonylation), and as deduced from the correlation data (Figs 2 and 3), it seems particularly significant 8 hours after loading, when Hcy reaches the maximum plasma peak. According to other reported findings,[21] a positive correlation exists between hyperhomocysteinemia and lipid oxidation (MDA). Furthermore, our data suggest that there may be an interesting oxidative effect on plasma proteins, possibly due to the generation of oxygen-reactive species in the presence of transitional metal ions during the oxidation of Hcy, in turn induced by the formation of disulfide

Table 3. Plasma Parameters in Controls (n = 4)

Parameter	Basal	4 h	6 h	8 h	24 h	χ^2 (4,4)
Hcy (nmol/mL)	18.36 ± 11.9	17.45 ± 12.6	18.9 ± 9.3	19.97 ± 11.6	17.97 ± 8.3	6.2*
PTOX (nmol/mg protein)	0.27 ± 0.05	0.33 ± 0.08	0.28 ± 0.17	0.24 ± 0.10	0.33 ± 0.15	3.59*
DIE (optic density units)	499 ± 169	522 ± 161	536 ± 176	508 ± 140	512 ± 146	3.392*
MDA (nmol/mL)	1.22 ± 0.83	1.16 ± 0.82	1.45 ± 1.10	1.30 ± 0.70	1.08 ± 0.50	4.911*

NOTE. χ^2 values are from the Friedman test for repeated-measures analysis.
*P = not significant.

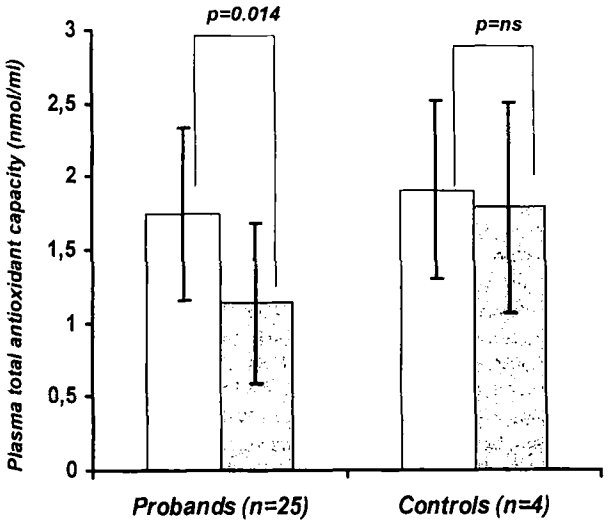

Fig 1. Total plasma antioxidant capacity in basal conditions (□) and 8 hours after a methionine load (■). Controls did not receive methionine. Intragroup comparison was made by paired Student's *t* test.

bonds between Hcy and lateral cysteine residuals of plasma proteins.[12,13]

It should be kept in mind that most plasma transitional metal in the circulation is variously chelated with proteins, which suggests that plasma proteins may be more exposed to Hcy-induced oxidative damage, and this may have important functional consequences. However, Hcy-induced oxidative damage would seem relevant only in cases of acutely induced hyperhomocysteinemia, a condition that is far from physiological. We found no significant correlations between Hcy basal levels and protein and lipid oxidative plasma markers, suggesting that only an acute increase in Hcy levels can induce an oxidative molecular alteration, the extent of which also seems to depend on the different basal antioxidant capacity available.

These observations suggest that diet (in terms of methionine

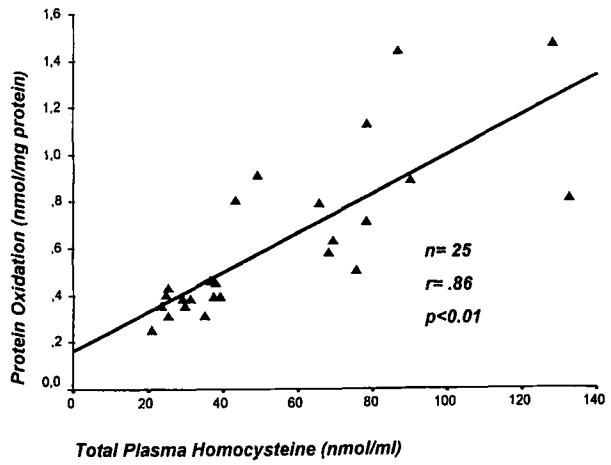

Fig 2. Correlation (Spearman's *r*) between plasma homocysteine and protein oxidation (measured as carbonylic groups) 8 hours after oral methionine intake.

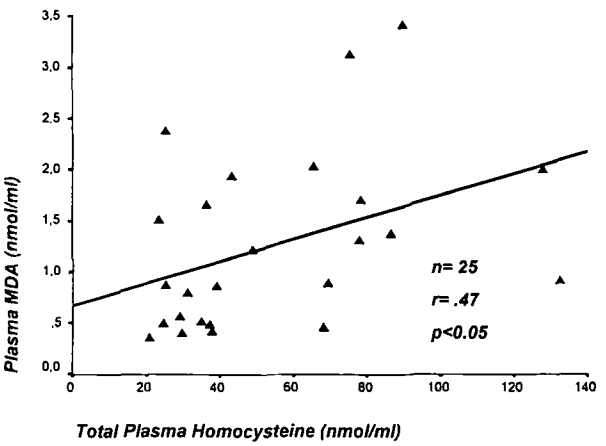

Fig 3. Correlation (Spearman's *r*) between plasma homocysteine and MDA 8 hours after oral methionine intake.

or antioxidant content) may play a role in influencing acute Hcy plasma levels and thereby altering the redox plasma balance and plasma content of oxidative-modified molecules.

To conclude, our data demonstrate that high total Hcy plasma levels acutely induced by oral administration of its precursor, methionine, increase lipid and, particularly, protein oxidation products in plasma. Since this effect is absent in the controls and the reduction in ANTOX (an indirect index of free-radical production) occurs only in the subjects exposed to methionine (and hence to hyperhomocysteinemia), our findings would support a pro-oxidant effect of high concentrations of Hcy acutely induced in vivo. The real importance of this effect in human physiology remains to be assessed, since hyperhomocysteinemia induced by oral methionine loading is far from a physiological condition. However, the role of the diet in inducing an acute postprandial increase in Hcy plasma levels, with its consequences for redox plasma postprandial balance, merits further investigation.

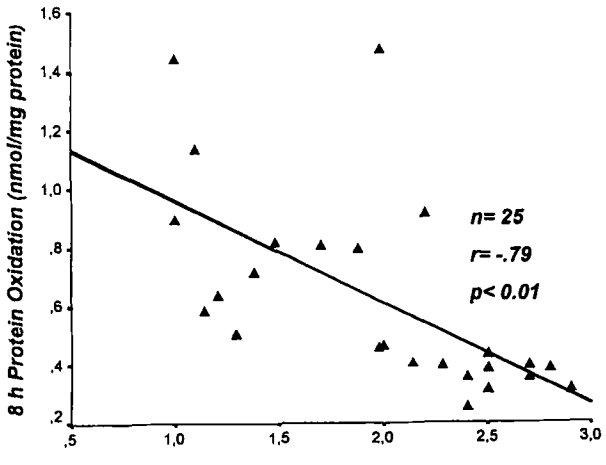

Fig 4. Correlation (Spearman's *r*) between basal plasma total antioxidant capacity and protein oxidation 8 hours after oral methionine intake.

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