Amino Acids (1996) 11: 99–104

Amino
Acids

Homocysteine and oxidative modification of plasma proteins

Short Communication

M. C. Mele and **E. Meucci**

Istituto di Chimica Biologica, Università Cattolica S. Cuore, Roma, Italy

Accepted August 14, 1995

Summary. The oxidative status of plasma proteins after incubation with elevated homocysteine levels has been examined in the presence and absence of transition metal ions. 200μM homocysteine alone does not provoke any loss of plasma thiols groups, but their oxidation significantly enhances as copper concentration increases. No plasma proteins carbonyl groups enhancement has been concurrently found.

The physiological relevance of the study is discussed in relationship with the metal-catalyzed oxidation system increment connected with age and nutritional deficiences.

Keywords: Amino acids – Homocysteine – Plasma proteins – Oxidative stress – Thiols groups – Atherosclerosis – Aging

Introduction

It is well known that elevated plasma homocysteine results in the damage of endothelial cells (Wall et al., 1980), an event considered to occur early in the process of atherogenesis (Starkebaum and Harlan, 1986). The toxic effect is mediated by oxygen radicals and H_2O_2 producted by the autooxidation of thiolcontaining amino acids (Olszewski and McCully, 1993). Oxygen radicals are known to interact with a variety of macromolecules, leading to lipid peroxidation, DNA strand breakage and a variety of changes in proteins, including thiols oxidation.

Bloom et al. (1992) have recently reported that plasma lipid peroxidation is not involved in the pathogenesis of hyperhomocysteinemia.

The present communication deals with the oxidative status of plasma proteins in the presence of elevated homocysteine amounts. The physiological level of homocysteine in human plasma is about 5–15μM (Ueland et al., 1993). Homocysteine concentrations occurring in plasma of untreated individuals with inherited homocystinuria are at least tenfold. More moderate elevations

have been found in 20–30% of individuals with coronary heart disease, peripheral vascular disease and stroke (Ueland and Refsum, 1989). We have chosen two amino acid levels: the greater one, 200μM, typical of subjects with homocystinuria, the lower one, 50μM, near to mild homocysteinemia found in subjects with premature cardiovascular diseases.

Materials and methods

DL – homocysteine, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma; 2,4-dinitrophenylhydrazine (DNPH) from B. D. H.; copper (II) sulphate $\cdot 5H_2O$ from Carlo Erba; guanidine HCl ultrapure from USB. All other chemicals used were of reagent grade.

Highly purified water (resistivity = 18 Mohm·cm) obtained through a Milli-Q water purification system (Millipore) was used for all solutions. Reagents were freshly prepared in 20 mM phosphate buffered saline (PBS), pH 7.4, and kept in a sealed tube on ice until used.

Blood was obtained by venipuncture after an overnight fast from healthy individuals, collected over heparin and immediately centrifuged (1500 × g for 10 min at +4°C); the plasma was pooled, stored at +4°C and used within six days.

Oxidation state of homocysteine and plasma thiol groups was determined according to Wayner et al. (1987), using DTNB, and the sulphydryl groups concentration calculated from the absorbance at 412 nm after 5 min of reaction (ε = 13,600 $M^{-1} \cdot cm^{-1}$).

Protein carbonyl groups assay was performed according to Reznick et al. (1992).

Erythrocytes lytic assays were performed according to Hider et al. (1983) on cells suspensions (5 and 10%) prepared from freshly drawn heparinized blood centrifuged for 10 min at 5,000 × g; plasma and buffy coat were removed by aspiration and the packed cells washed three times in cold saline. At various intervals of red bood cells incubation at 37°C with homocysteine, samples were withdrawn and centrifuged for 30 s on a Eppendorf Microfuge. 0.1 ml of the supernatant was diluted to 1 ml with saline and its absorbance monitored at 578 nm.

Absorbance was measured with a Hewlett-Packard 8450 A UV/Vis spectrophotometer equipped with a cuvette stirring apparatus and a constant temperature cell holder.

Measurements of pH were made with a PHM 84 Research pHmeter (Radiometer); the electrode response was corrected for temperature.

Results and discussion

Influence of copper on homocysteine oxidation

In 20 mM PBS, pH 7.4, containing only trace amounts of cationic metals, 50 and 200μM homocysteine lost 45 ± 5% and 30 ± 3% of thiols groups (n = 3), respectively, after 90 min exposure to air at 37°C. However, the addition of increasing amounts of Cu(II) in PBS caused increased oxidation of the thiols groups. 50μM homocysteine was completely oxidized in about 30 min in the presence of only 10μM copper sulphate; 200μM homocysteine was completely oxidized in about 60 and 30 min in the presence of 10 and 20μM copper ions, respectively (inset of Fig. 1).

Our results are in perfect agreement with the finding of Starkebaum and Harlan (1986) that micromolar amounts of copper catalyze an oxygen-

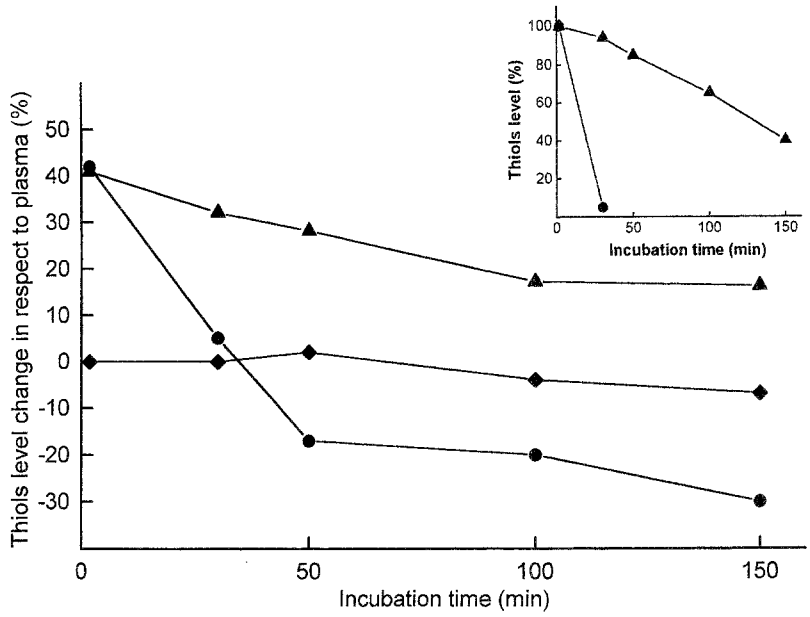

Fig. 1. Loss of total thiols in plasma containing homocysteine with or without redox-active metal ions addition. At various intervals plasma samples were withdrawn during incubation at 37°C with 200μM homocysteine in absence (▲) and presence (●) of 20μM copper (II) and analyzed for thiols groups as described under "Materials and methods". Results are reported as difference (%) in respect to thiols concentration of plasma alone. Copper sulphate was added before homocysteine addition. Oxidation of plasma in presence of 20μM copper (II) without homocysteine addition is also shown (◆). Thiols level of plasma alone did not change during incubation. *Inset*: Oxidation of 200μM homocysteine in 20mM PBS, pH 7.4 in absence (▲) and presence (●) of 20μM copper (II). Data in figure are representative of a typical result obtained in three separate experiments

dependent oxidation of homocysteine. So the sulphydryl group of homocysteine is found to act with cupric ions in a metal-catalyzed oxidation system to generate hydrogen peroxide, oxygen radicals and homocysteinyl radicals. These reactive species may interact with plasma macromolecules or cause injury to cells.

Effect of homocysteine on plasma proteins

The addition of 50 and 200μM homocysteine to fresh or outdated plasma caused an initial thiols groups enhancement of about 8 ± 2 and 41 ± 5%, respectively (Table 1). In these conditions total sulphydryls groups loss after 90min incubation can be ascribed to the amino acid oxidation, due essentially to plasma ceruloplasmin (Starkebaum and Harlan, 1986), as well as to the binding of homocysteine to plasma proteins. In fact, it is well known that a major fraction of total homocysteine in plasma from normal subjects is complexed with serum albumin, probably via a disulfide bond (Ueland et al., 1993). The binding seems to be saturable and this implies that the free fraction of homocysteine increases more than protein-bound homocysteine does when

Table 1. Influence of transition metal ions on homocysteine and plasma proteins thiols oxidation

Condition	Thiols enhancement[a]	Thiols oxidation[b], Copper (II) added, μM			
		0	10	20	40
Plasma alone	0	1 ± 1	2 ± 1	10 ± 4	17 ± 7
Plasma + 50μM Homocysteine	8 ± 2	7 ± 1	8 ± 2	11 ± 2	ND
Plasma + 200μM Homocysteine	41 ± 4	21 ± 6	32 ± 3	44 ± 5	49 ± 3

[a] thiols enhancement (%) in respect to plasma alone measured immediately after homocysteine addition. [b] thiols loss (%) after 90 min incubation in respect to initial time. Samples were withdrawn after incubation at 37°C with or without 50 and 200μM homocysteine and copper ions and analyzed for thiols groups as described under "Materials and methods". Copper sulphate was added before homocysteine addition. Values are means ± SD of three separate determinations in duplicate. *ND* not determined.

total amino acid is markedly increased. *In vitro* redistribution is found to take place within 24 h at room temperature (Ueland et al., 1993). In absence of metals addition, finall detectable amount of total thiols groups was equal or greater than initial plasma thiols groups level after incubation with 50 and 200μM homocysteine, respectively (Fig. 1).

When micromolar amounts of copper sulphate were added to the plasma, a much greater thiols groups loss was observed after incubation (Table 1). The finding could well reflect an increase in the production of H_2O_2. Even though H_2O_2 is itself a weak oxidizing agent, its toxic effects are likely to be mediated by the $HO^{\bullet}$ which is readily formed in the presence of transition metal ions and may produce oxidative damages to biological macromolecules. In particular at high homocysteine and redox-active metal ion concentrations the final thiols groups amounts were much lower in respect to those found in plasma alone. 50μM homocysteine caused a low decrease (3–4%) in plasma thiols groups only in the presence of at least 20μM copper ions, whereas 200μM homocysteine provoked a plasma thiols groups oxidation of about 6 ± 1%, 20 ± 3% and 33 ± 3% in the presence of 10, 20 and 40μM copper (II), respectively (Fig. 1). Starkebaum and Harlan (1986) did not report any oxidation of serum sulphydryl groups, but it is worthnoting that their experiments were performed after a very short incubation period (i.e., 10 min only).

Even considering the oxidative effect of copper alone (Table I), our results clearly indicate a loss of plasma thiols groups due to the metal-catalyzed system at amino acid and redox-active metal ions concentrations found *in vivo* in different pathological conditions (Olszewski and McCully, 1993).

Furthermore, our studies suggest that protein sulphydryls could serve as sacrificial antioxidants, as well as being targets for oxidative damage. This argument is supported by the carbonyl-group assay, which provides a convenient technique for detecting and quantifying another protein oxidative modification. No protein carbonyl groups increase was found after 90 min incubation with 50 and 200μM homocysteine, even in the presence of 40μM

copper (II) (data not shown), so confirming the specificity of the thiols oxidative attack.

Effect of homocysteine on erythrocytes

Homocysteine (0.25 to 3.0 mM) could not lyse red blood cells neither in the absence nor in the presence of 20 μM copper ions, even after 6 h of incubation (data not shown). On the contrary, endothelial cells were seen to lyse after 4 h of incubation with homocysteine plus copper (Starkebaum and Harlan, 1986). At the present it is not clear the reason of this difference; nevertheless the data elucidate that injury to endothelial cells in very specific.

Conclusions

Homocysteine is an amino acid of special interest for its claimed link with the development of atherosclerosis. Vascular occlusive disease is the primary cause of premature death in homocystinuria and mild homocysteinemia has been found to be an independent risk factor in coronary, cerebral and peripheral atherosclerosis. Age, menopausal status and nutritional deficiences of folate, pyridoxine or cobalamin increase the basal plasma level of homocysteine. Redox-active metal ions concentrations increase in many pathological conditions by decompartmentalisation of metal-complexes. These evidences, together with our results, raise the possibility that this physiological metal-catalyzed system can cause oxidative modifications of plasma proteins in addition to the damages already known.

Caution is necessary in trying to extrapolate *in vitro* observation to an *in vivo* situation. Our communication clearly suggests the need for in-depth analyses of oxidative status of plasma proteins isolated from subjects with high homocysteine levels.

Nevertheless the data confirm the findings of Radi et al. (1991) on xanthine oxidase-derived oxidants that plasma proteins are a more sensitive and specific indicators of oxidant stress than assessment of lipid oxidation byproducts. Moreover, the present study supports a strategy for control of homocysteinemia by micronutrients, such as folate, which could ameliorate the metabolic abnormalities characteristic of hyperhomocysteinemia and contribute to human health and longevity.

References

Blom HJ, Engelen DPE, Boers GHJ, Stadhouders AM, Sengers RCA, de Abreu R, TePoele-Pothoff MTWB, Trijbels JMF (1992) Lipid peroxidation in homocysteinaemia. J Inher Metab Dis 15: 419–422

Hider RC, Khader F, Tatham AS (1983) Lytic activity of monomeric and oligomeric melittin. Biochim Biophys Acta 728: 206–214

Olszewski AJ, McCully KS (1993) Homocysteine metabolism and the oxidative modification of proteins and lipids. Free Rad Biol Med 14: 683–693

Radi R, Bush KM, Cosgrove TP, Freeman BA (1991) Reaction of xanthine oxidase-derived oxidants with lipid and protein of human plasma. Arch Biochem Biophys 286: 117–125

Reznick AZ, Cross CE, Hu M-L, Suzuki YJ, Khwaja S, Safadi A, Motchnik PA, Packer L, Halliwell B (1992) Modification of plasma proteins by cigarette smoke as measured by protein carbonyl formation. Biochem J 286: 607–611

Starkebaum G, Harlan JM (1986) Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine. J Clin Invest 77: 1370–1376

Ueland PM, Refsum H (1989) Plasma homocysteine, a risk factor for vascular disease: plasma levels is health, disease and drug therapy. J Lab Clin Med 114: 473–501

Ueland PM, Refsum H, Stabler SP, Malinow MR, Andersson A, Allen RH (1993) Total homocysteine in plasma or serum: methods and clinical applications. Clin Chem 39: 1764–1779

Wall RT, Harlan JM, Harker LA, Striker GE (1980) Homocysteine-induced endothelial cell injury in vitro: a model for the study of vascular injury. Thromb Res 18: 113–121

Wayner DDM, Burton GW, Ingold KU, Barclay LRC, Locke SJ (1987) The relative contributions of vitamin E, urate, ascorbate and proteins to the total peroxyl radical-trapping antioxidant activity of human blood plasma. Biochim Biophys Acta 924: 408–419

Authors' address: Dr. E. Meucci, Istituto di Chimica Biologica, Università Cattolica S. Cuore, Largo F. Vito 1, I-00168 Roma, Italy.

Received May 18, 1995

Verleger: Springer-Verlag KG, Sachsenplatz 4–6, A-1201 Wien. – Herausgeber: Prof. Dr. Gert Lubec, Universitäts-Kinderklinik, Währinger Gürtel 18–20, A-1090 Wien. – Redaktion: Sachsenplatz 4–6, A-1201 Wien. – Hersteller: Satz und Umbruch: Best-Set Typesetter Ltd., Hong Kong; Offsetdruck: Novographic, Ing. W. Schmid, Maurer-Lange-Gasse 64, A-1238 Wien. – Verlagsort: Wien – Herstellungsort: Wien. – Printed in Austria.