

Biochemical Model Reactions on the Prooxidative Activity of Homocysteine

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The sulfur amino acid homocysteine has recently been addressed as marker for vessel damaging and atherosclerotic dispositions. The atherogenic index has been correlated with the one of cholesterol and is significantly higher in cholesterinemic as compared to normal lipidemic persons. In the present communication biochemical model reactions are presented indicating the prooxidative activity of homocysteine where a cooperative effect with the transition-metals copper and iron is indicated.

Introduction

The non-proteinogenic sulfur amino acid homocysteine is an important connecting member in the intermediary metabolism of the essential sulfur amino acids methionine and cysteine and thus also important for the homeostasis of glutathione. This sulfur amino acid cycle is also the source of adenosyl-methionine which in turn is functioning in a broad spectrum of cellular synthesizing capacities. The catalysis of this cycle is dependant on the presence of the vitamins B₆, B₁₂ and folic acid. Due to lack of these vitamins or genetic deficiencies in the regulation of the homocysteine concentrations such as homocystinuria, atherogenic symptoms are frequently observed already before the third life decade [1]. In healthy persons the average homocysteine concentrations are at approximately 10.5 nmol/ml blood serum. Stampfer *et al.* [2] recently documented in a prospective study of plasma homocysteine with regard to the risk of myocardial infarction in U.S. physicians that the average increase of approximately 1 nmol/ml homocysteine clearly correlated with an increased risk of myocardial infarction. In hypercholesterinemic patients the homocysteine content of the LDL-fraction was also significantly increased [3]. The authors thus suggested that homocysteine determination may be taken as a marker for atherogenic tendencies and connected risk assessments. On the other hand it has recently been documented that the oxidation of LDL for example by copper salts

leads to modifications of certain epitopes of this lipoprotein which in turn are connected with an uncontrolled uptake of these molecules through the so-called scavenger receptor of macrophages finally yielding plaques in the intima of the blood vessels [4].

The present communication describes model reactions documenting the prooxidative activity of homocysteine thus increasing the oxidative stress in the blood vessels [5, 6].

As model reactions we tested the disturbance of membrane properties shown as

- 1) hemolysis of erythrocytes;
- 2) the damage of the Na⁺/K⁺-ATPase as an essentially enzyme controlling the cellular ion homeostasis and
- 3) the oxidative fragmentation of a small sulfur-keto-carbonic acid (keto-methiolbutyric acid, KMB) as an immediate relative of methionine.

Materials and Methods

Chemicals and biochemicals

ATP, Na⁺/K⁺-ATPase, DL-Cysteine, DL-Homocysteine, EDTA, FeCl₃, FeSO₄, KMB, MgCl₂ Trichloroacetic acid and Trizma base were purchased from Sigma Chemicals Co. (Munich, Germany). Ammoniumheptamolybdat, CuSO₄, KCl, NaCl, NaH₂PO₄, Na₂HPO₄ and H₂SO₄ were from Merck (Darmstadt, Germany). N₂, H₂ and synthetic air were obtained from Messer-Griesheim (Mannheim, Germany). Human blood conserves (erythrocyte concentrates) were purchased from "Rotes Kreuz" (Munich, Germany).

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Methods

Hemolysis of erythrocytes

The erythrocytes were washed by centrifugation (3000 U/min; 4 °C) three times with a 1:1 mixture of PBS and 0.9% NaCl. The cells were then suspended in the PBS/NaCl-solution to give a final Hb-concentration of 15 g%. All chemicals used in the hemolysis experiment were dissolved in PBS.

1.0 ml of erythrocytes were incubated at 37 °C in the dark for 120 min with homocysteine (0.5 mM) and FeSO₄/EDTA or CuSO₄/EDTA (0.5 mM or 1.0 mM). The final reaction volume of 2.0 ml was obtained by the addition of PBS. After the incubation period the red cells were centrifuged at 2400 U/min for 5 min and the relative hemoglobin content in the supernatant was determined with a Uvikon spectrophotometer (type 930) at 540 nm. As a reference we used the extinction of the supernatants of controls without incubation.

Na⁺/K⁺-ATPase

The Na⁺/K⁺-ATPase was preincubated for 30 min at 37 °C in the dark in a reaction mixture containing 0.5 ml Tris-buffer, 0.2 M, pH 7.4; 50 µl salt mix (NaCl 2.5 M; KCl 0.6 M; MgCl₂ 90 mM); 50 µl Na⁺/K⁺-ATPase (1 U/ml); ± 50 µl FeSO₄ (0.5 mM); ± 50 µl EDTA (0.5 mM); ± 50 µl homocysteine or cysteine at various concentrations; aq. dest. *ad* 0.95 ml.

After 30 min the enzyme-catalyzed ATP-hydrolysis was started by the addition of 50 µl ATP (40 mM in Tris-buffer) and the assay was again incubated for 30 min in the dark. The enzyme-reaction was stopped by the addition of 0.75 ml TCA (20%) and the tubes were centrifuged at 3000 U/min for 5 min. 0.5 ml of the supernatant were pipetted to 0.5 ml aq. dest. and 0.5 ml Tausky-Shorr-reagent and the extinction was measured after 10 min at 660 nm.

KMB-fragmentation

The assays (reaction volume 2.0 ml, containing phosphate buffer, pH 7.4; 0.1 M; KMB 1.0 mM; FeCl₃ 50 µM; EDTA 50 µM; homocysteine at various concentrations) were incubated in flasks of known volume and sealed with gas-tight rubber caps. After 120 min of incubation at 37 °C in the

dark 1.0 ml of gas was taken from the headspace of the tubes with a gas-tight syringe and analyzed by gas chromatography (Varian 3300; 1/8" × 1 m 60/80 deactivated aluminea column; injector temperature 80 °C; column temperature 60 °C; detector temperature 225 °C).

Results

Hemolysis of erythrocytes

As shown in Fig. 1 neither homocysteine nor 0.5 mM CuSO₄ alone yield hemolysis of erythrocytes as compared to the artefactual spontaneous hemolysis in the controls. The combination of Cu²⁺ and homocysteine however yields significant hemolysis after 120 min of incubation. The hemolysis by equimolar homocysteine/Fe²⁺-combinations is small as compared to the one catalyzed by Cu²⁺.

Inactivation of Na⁺/K⁺-ATPase

ATPases are enzymes which are responsible for maintaining the ion-balances by actively pumping Na⁺, K⁺ or Ca²⁺ through cellular membranes. Damage of these enzymes has been shown to result in ion-imbalances and thus rapid decay of membraneous components such as lipids. As a final result cellular function is strongly impaired. This inactivation of Na⁺/K⁺-ATPase is achieved by the Fenton-type oxidants Fe²⁺/EDTA (Fig. 2). This activity is enhanced by addition of 50 µM homo-

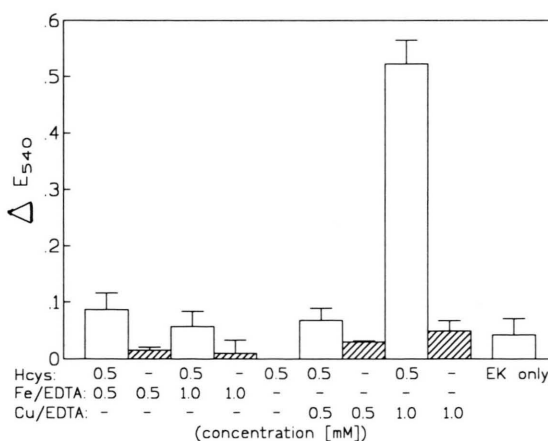


Fig. 1. Hemolysis of erythrocytes by DL-homocysteine and Fe²⁺ or Cu²⁺. (Incubation for 120 min at 37 °C in the dark.)

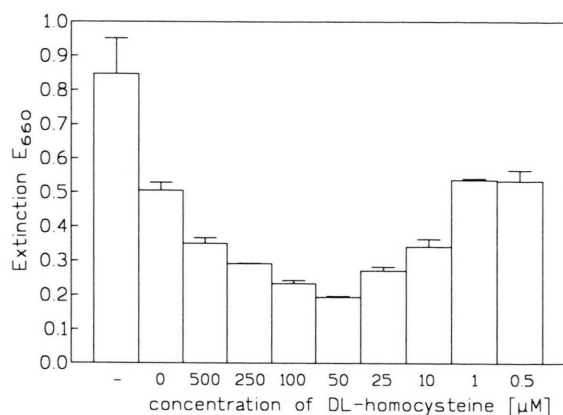


Fig. 2. Inactivation of Na⁺/K⁺-ATPase by 25 μM FeSO₄/EDTA: effect of various concentrations of DL-homocysteine. (-: ATPase without FeSO₄/EDTA.)

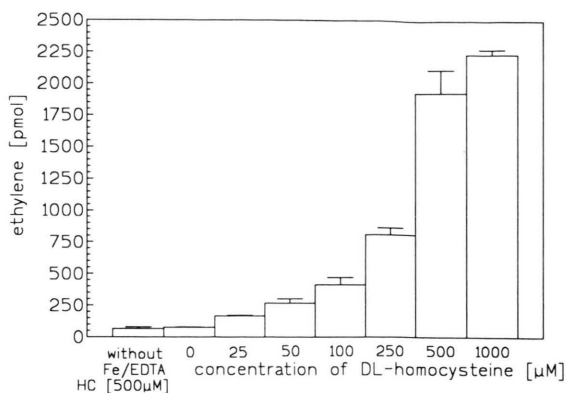


Fig. 3. Production of ethylene after fragmentation of KMB by 50 μM FeCl₃/EDTA: effect of various concentrations of DL-homocysteine. (Incubation for 120 min at 37 °C in the dark.)

cysteine. Higher concentrations of homocysteine yield in a diminution of the damaging effect. Almost identical results have been obtained by Issels and Wilmanns [7] investigating the damaging kinetics of clonogeneous CHO-cell cultures by cysteamine at 37 °C. These authors addressed this effect as a paradoxical diminution of toxicity at higher concentrations of the thiol. Very similar effects have also been shown by Beach and Giroux [8] investigating lipid peroxidation promoted by Fe³⁺ and ascorbate.

Fragmentation of keto-methiolbutyric acid (KMB)

KMB, the transamination product of methionine has been used in numerous biochemical experiments as an excellent indicator for biologically relevant oxidative stress situations both *in vitro* and on a cellular platform. As shown in Fig. 3 KMB is fragmented yielding ethylene in an Fe³⁺/homocysteine-dependent reaction. This effect is visible at 50 μM Fe³⁺ and 25 μM homocysteine. Increasing concentrations of homocysteine yield a further stimulation of ethylene formation. In analogy to other oxygen activating systems such as xanthin oxidase or diaphorase-coupled quinone redox-cycling [5, 6, 9–12] the formation of a strong oxidant of the OH-radical type is indicated by these effects where even at 1 mM concentrations of homocysteine no saturation is observed.

Comparison of homocysteine- and cysteine-mediated oxygen activation

Cysteine is present in living aerobic cells and in the blood in concentrations in the range of 100 μM and higher [15]. Thus cysteine concentrations in the blood are approximately ten times higher than those of homocysteine. If the effect of cysteine and homocysteine on ATPase is compared it is clearly visible that a damage of ATPase at concentrations of 50 and 100 μM of cysteine is not observable; in contrast to 100 μM homocysteine which supports the Fe²⁺/EDTA-mediated damage of ATPase,

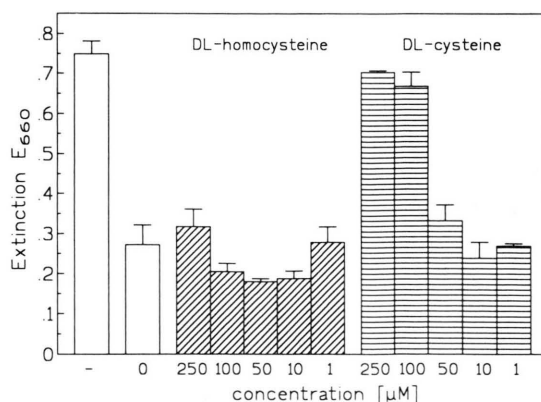


Fig. 4. Inactivation of Na⁺/K⁺-ATPase by 25 μM FeSO₄/EDTA: comparison of the effects of DL-homocysteine and DL-cysteine. (-: ATPase without FeSO₄/EDTA.)

100 μM cysteine clearly reverse the Fe^{2+} /EDTA-mediated damage. The even stronger damaging effect of 50 μM homocysteine is not observable with 50 μM cysteine (Fig. 4).

Discussion

Atherosclerosis is an extremely complex disease which cannot be described by just one simple theory [14]. Cholesterol and LDL and/or HDL have been used as biochemical markers for atherogenic potentials in man and animals. More recently homocysteine has also been brought into context with atherosclerotic phenomena [1, 3, 15, 16]. This interest in homocysteine metabolism stems from the inborn error of homocysteine metabolism called homocysteinemia where the remethylation and transsulfuration of homocysteine is incoordinated and partially interrupted [15]. Since homocysteinemic persons frequently suffer from atherogenic phenomena it has been assumed that homocysteine increases in the bloodstream might be directly connected with pathophysiological conditions resulting in vascular diseases. It has also become clear in the last couple of years that one principle of atherogenic activities is the oxidation of the apolipoprotein B of LDL yielding a lipoprotein product which is no longer taken up by macrophages *via* the regular LDL metabolizing pathway: oxidized LDL is taken up by the scavenger receptor which is not back-regulated and thus yields uncontrolled uptake of these lipoproteins. The result of this activity is the formation of foam cells and finally plaques in the intima.

In the present paper we asked the question whether homocysteine exhibits a prooxidative potential in a concentration range observable in homocysteinemic or atherosclerotic persons. As shown in Fig. 1, 2 and 3 homocysteine in the presence of either copper or iron exhibits a significant prooxidative potential as shown by the hemolysis of erythrocytes, oxidation of KMB yielding ethylene or damage of Na^+/K^+ -ATPase. Sandhu *et al.* [17] recently demonstrated that peroxyl radical-mediated hemolysis may be connected with the

damage of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase and the oxidation of GSH which clearly precede the hemolytic event. The damage of Na^+/K^+ -ATPase shown in our experiment exhibits a bell-shaped concentration dependence of homocysteine: higher concentrations of homocysteine seem to protect ATPase from oxidative damage. Similar effects have been shown for cysteamine [7] and iron/ascorbate [8]. Cysteine in the same range of concentrations and especially at the cellular concentration level of this amino acid does not exhibit this damaging effect on ATPase (Fig. 4).

From our result we can conclude that a) homocysteine (in the concentration range between 10 and 50 μM) like other thiol compounds exhibits an expressed prooxidative effect which is only visible in the presence of either iron or copper as transition-metal ions; b) oxidative damage on a cellular niveau (erythrocytes) or on enzymes (ATPase) or small molecules like KMB clearly support the view that homocysteine exhibits a broad spectrum of potentially damaging activities concerning various levels of pathophysiological events. Several antioxidants like α -tocopherol inhibit this damaging effect (data not shown).

Thus the clinically documented heterogenic effect of homocysteine may be due to its prooxidative activity in the presence of transition-metal ions. The results are also in agreement with the nowadays generally accepted assumption that atherogenic processes in the blood vessels are oxidative where antioxidants may be important for preventing the effects. Since the status of the vitamins B_6 , B_{12} and folic acid are intimately connected with homocysteine metabolism, a sufficient supplementation with these vitamins may be a very basic prerequisite for preventing atherogenic potentials and several other diseases connected with homocysteine. The contrasting effects of cysteine and homocysteine concerning ATPase damage are currently under investigation.

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