Homocysteine-Induced Oxidative Damage: Mechanisms and Possible Roles in Neurodegenerative and Atherogenic Processes

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Increased blood plasma concentrations of the sulphur amino acid homocysteine ("homocysteinemia") have been brought into context with neurodegenerative and arteriosclerotic symptoms and diseases.

We recently reported on biochemical model reactions on the prooxidative activity of homocysteine including the desactivation of Na^+/K^+-ATPases and hemolysis of erythrocytes (Preibisch *et al.*, 1993). In this communication we extend our model reactions including the oxidation of methionine, metabolization of pyridoxalphosphate and dihydroxyphenylalanine, desactivations of transaminases and peroxidation of low density lipoprotein.

Introduction

The biosynthesis of methyl group donors such as S-adenosyl-methionine (SAM) and of cysteine (Cys) are essential for the synthesis of methylated elements of cell membrane structures such as phosphatidylcholine and of antioxidants such as S-methyl-α-ketobutyric acid (KMB), taurin and glutathione. These central functions are warranted by the homeostasis of methionine metabolism keeping the overall cellular antioxidative system at high fidelity. If this system is out of balance, a vast amount of pathological processes may be induced mainly concerning nervous disorders and atheromatous developments. Homocysteine (HC) as one member in this amphibolic process, however, posseses prooxidative functions which are biochemically distinct to the reactivities of cysteine (Preibisch *et al.*, 1993; Preibisch and Elstner, 1994). As recently reported, the pyridoxalphosphate-glutamate Schiff-base has strongly antioxidative properties preventing damage by organic hydroperoxides (Meyer *et al.*, 1992). In earlier papers (Konze and Elstner, 1976; Saran *et al.*, 1980; Youngman *et al.*, 1982; Youngman and Elstner, 1985; Elstner *et al.*, 1980) we reported that methionine in the presence of pyridoxalphosphate is fragmented by certain strong oxidants yielding ethene. This reaction proved as a sensitive indicator for the production of OH-type radicals. The transamination product of Met, S-methyl-α-ketobutyric acid (KMB) is a much less specific but very sensitive indicator for the generation of a wide variety of oxygen radicals including Cys or HC in the presence of copper or iron ions (Preibisch *et al.*, 1993). In the absence of PyP no ethene is released from Met by certain biological systems such as membraneous oxidoreductases in the presence of autoxidizable redox cyclers or by NAD(P)H oxidases. Methionine (Met) in combination with pyridoxalphosphate (PyP) or KMB act as scavengers of strong oxidants such as the $OH^{\cdot}$-radical. Thus, the conversion of homocysteine into methionine catalyzed by cobalamine (vitamin B_{12}) in cooperation with folic acid clearly shifts a prooxidative towards an antioxidative situation. Vitamin B_6 (PyP) both catalyzes the synthesis of KMB as well as the reactivity of methionine as radical scavenger. Thus, the vitamins folic acid and B_{12} indirectly act as antioxidants by removing homocysteine and producing methionine, whereas PyP both in the presence and absence of methionine is an effective antioxidant. In this communication we present experimental evidence that HC in the presence of copper or iron ions (see Discussion)

Abbreviations: Cys, cysteine; HC, homocysteine; HCTL, homocysteinethiolactone; Cu, copper; KMB, 4-methylthio-2-oxobutanoic acid; Met, methionine; PyP, pyridoxal phosphate; LDL, low density lipoprotein; TA, transaminase; DOPA, dihydroxyphenylalanine.

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destroys or alters biomolecules such as transaminases (TA), dihydroxyphenylalanine (DOPA) or low density lipoprotein (LDL) which are actively involved in the above mentioned diseases. Homocysteine and methionine metabolism is tightly connected and plays a crucial role in a large variety of diseases. An outline of this connection including the mentioned damaging reactions as well as their counterbalance is presented in Fig. 1.

Materials and Methods

Chemicals and Biochemicals

Chemicals were obtained from SIGMA Chemical Co. (Munich) in the greatest available purity.

Likewise, the enzymes glutamic-oxalacetic transaminase EC 2.6.1.1 from porcine heart G-7005, glutamic-pyruvic transaminase EC 2.6.1.2 from porcine heart G-8255, malic dehydrogenase EC 1.1.1.37 from *Thermus flavua* M-7032, lactic dehydrogenase EC 1.1.1.27 from porcine heart L-2625 and polyphenoloxidase (tyrosinase) EC 1.14.18.1 from mushroom T-7755 were purchased from SIGMA (Munich).

Met/PyP-system

Met and PyP were allowed to react at room temperature in 0.2 M phosphate-bufferd solution (pH 7.4) for 10 min forming the Schiff-base. Then, Fe^{2+} or Cu^{2+} ions ($FeCl_2 \cdot 4H_2O$ or $CuSO_4 \cdot 5H_2O$ dissolved in aqua bidest.) and H_2O_2 were added. Ethene production was measured gaschromatographically after a 45-min incubation at 37 °C. The final concentrations, unless otherwise indicated in the legends, were 1.0 mM for Met and PyP, 1.0 mM for the metal ions and 0.5 mM for the hydrogen peroxide.

To test the influence of Cys, HC and HCTL on methionine fragmentation the substances (0.01 up to 2 mM final concentration) were added before reaction start with metal ions and H_2O_2 solution.

Assays for transaminase activity

For spectrophotometric determination of transaminase activities the procedures of Mavrides (1987) and SIGMA (quality control test procedure 10/'93; available upon request) were performed in a slightly modified manner: The substrates for the transaminase and the dehydrogenase were pipetted into half micro disposable cuvettes and the reaction was started by adding transaminase solution. The final concentrations of the glutamic-oxalacetic transaminase assay were 50 mM L-aspartate, 10 mM α-ketoglutarate, 0.2 mM β-NADH, 2.4 U malic dehydrogenase and 0.3 U transaminase. For the glutamic-pyruvic transaminase assay 200 mM D,L-alanine, 10 mM α-ketoglutarate, 0.2 mM β-NADH, 7.5 U lactic dehydrogenase and 0.1 U transaminase were added. (Unit definition: One International Unit (U) is defined as that amount of enzyme that will form 1 μmol of glutamate per minute at pH 7.5 and 25 °C.). After stirring the decrease in absorbance at 340 nm was recorded

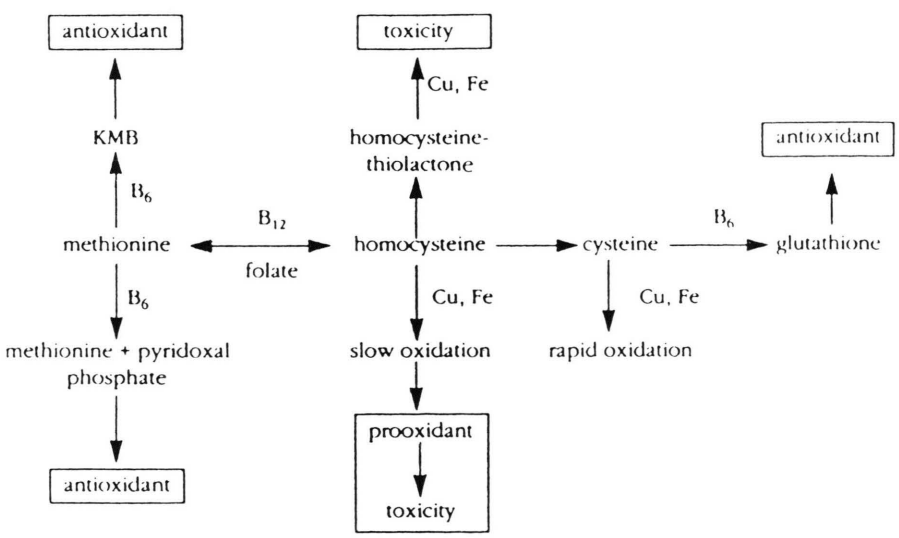

Fig. 1. Methionine-homocysteine metabolism.

for approximately 15 min to obtain the maximum linear reaction rate (ΔA/min = activity).

To study the influence of the test substances (Cys, HC, HCTL) on enzyme activity a preincubation of the transaminases with the sulphur compounds at 37 °C for 15 min preceeded the addition to the reaction mixture. Activities were compared as percentages of control activity.

Reactions with o-quinone products from PPO-catalyzed dihyroxyphenylalanine (DOPA) oxidation

A mixture containing 9.6 mM DOPA, 0.8 mM Cys/HC/HCTL respectively and 783 U polyphenoloxidase in 0.2 M phosphate buffered solution (pH 6.0) was allowed to react 2.5 – 5 – 10 – 20 – 30 – 45 – 60 min, respectively, then the reaction was stopped by adding an equal volume of methanol. Particles were removed by a 10 min centrifugation at 1000×*g* in an Eppendorf-centrifuge. After dilution 1 : 5 with the reaction buffer, HPLC analysis was performed. A reversed phase column Hypersil ODS (particle size 5 µm, pore size 100 Å) was used. The flow rate was 1 ml 50 mM phosphate buffer pH 2.4 / min and products were detected at 254 nm.

Oxidation of low density lipoprotein

LDL from human venous blood was subjected to oxidation by 2.5 and 5 µM Cu^{2+} respectively.

Dieneconjugation was measured spectrophotometrically by monitoring increase in absorbance at 234 nm, changes in electrophoretic mobility were determined by agarose gel electrophoresis (according to Cooper, 1983).

Results

The combination of both Met and PyP is apparently producing an antioxidant rapidly reacting with the potent oxidant of the OH· type, including transition metal-oxygen complexes of the "Fenton-type" i.e. Fe^{2+} in the presence of a peroxide. We assayed this reaction with respect to the effects of iron or copper ions, which are under increasing discussion as initiators and/or cofactors of the developments of neurodegenerative (Youdim and Lavie, 1994) and atherosclerotic diseases (Meyer *et al.*, 1992; Kögl *et al.*, 1994; Lupo *et al.*, 1994), where the sulphur containing amino acid, methionine, seems to be in the focus of such oxidative destructions (Preibisch and Elstner, 1994; Gilman *et al.*, 1993).

1) Reactions with methionine and pyridoxalphosphate

In the presence of either $iron^{2+}$ or $copper^{2+}$ ions, the release of ethene from methionine was tested as to the effects of cysteine, homocysteine, or homocysteine thiolactone.

As shown in Fig. 2, in the presence of hydrogen peroxide and either iron or copper, increasing amounts of ethene are released from increasing concentrations of Met in the test system. This reaction is inhibited by PyP in the copper system and to a much lesser extent in the slower reacting iron system (Fig. 2).

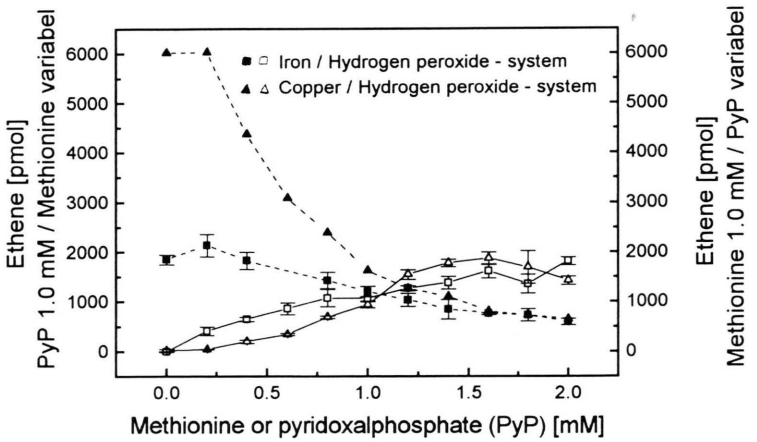

Fig. 2. Release of ethene from methionine: dependence on methionine (open symbols) or on pyridoxalphosphate (closed symbols) concentration. H_2O_2 0.5 mM; Fe^{2+} 1.0 mM (squares) or Cu^{2+} 1.0 mM (rectangles). Either methionine or pyridoxalphosphate vary in concentration the other being constant 1.0 mM.

Increasing concentrations (0.01 to 2 mM) of the above mentioned sulfhydryl compounds (Cys, HC, HCTL) to various extents inhibit the transitionmetal-hydrogenperoxide driven ethene production from Met (Figs 3a and 3b).

Cys and HC can interact with PyP forming thiazolidine and thiazine complexes (Schonbeck *et al.*, 1975; Cooper, 1983), optically visible as the disappearance of the absorption at 386 nm and the formation of a new absorption band at 328 nm and 326 nm, respectively (Figs 4a and 4b).

2) Reactions with transaminases

The reactions with PyP may reflect the sensitivity of certain transaminases towards oxidation by either copper ions or thiol compound – copper combinations as demonstrated in Figs 5 and 6: Glutamic-oxalacetic transaminase is only inhibited by high concentrations of HCTL (Fig. 5) while glutamic-pyruvic transaminase is more or less sensitive towards Cu (Fig. 6a) as well as Cys and HCTL. Cu-inhibition is reversed by HCTL and Cys while high concentrations (> 10 mM) of Cys or HCTL are inhibitory (Fig. 6b).

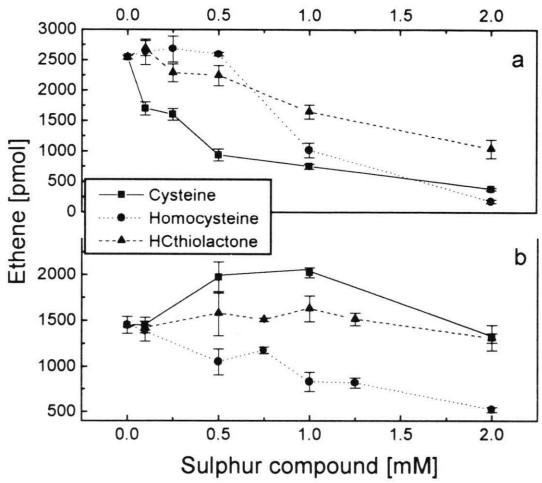

Fig. 3. Effects from various amounts of cysteine, homocysteine or homocysteinethiolactone on the Cu^{2+}-hydrogenperoxide-driven (a) and on the Fe^{2+}-hydrogenperoxide-driven (b) ethene production from methionine. H_2O_2 0.5 mM; Met, PyP, Cu^{2+} or Fe^{2+} 1.0 mM.

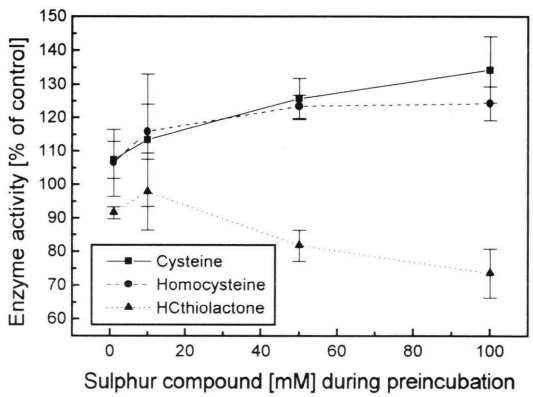

Fig. 5. Activity of glutamic-oxalacetic transaminase (0.3 U) after preincubation (15 min, 37 °C) with various amounts of cysteine, homocysteine or homocysteinethiolactone: 1; 10; 50; 100 mM.

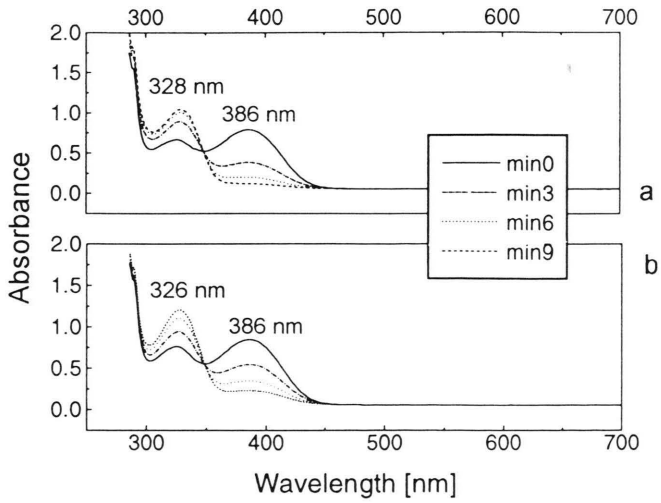

Fig. 4. Formation of a cyclic thiazolidine derivative from pyridoxalphosphate and cysteine (a) and of a cyclic thiazine derivative from pyridoxalphosphate and homocysteine (b). PyP 180 μM; Cys or HC 2.5 mM; 37 °C, pH 7.4.

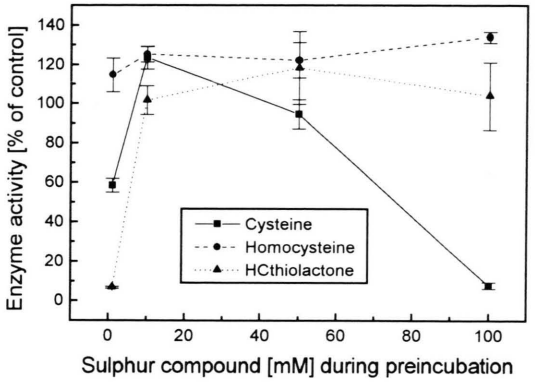

Fig. 6a. Activity of glutamic-pyruvic transaminase (0.1 U) after preincubation (15 min, 37 °C) with various amounts of cysteine, homocysteine or homocysteinethiolactone: 1; 10; 50; 100 mM in the presence of Cu^{2+} 5 μM. The activity of the enzyme after preincubation with 5 μM Cu^{2+} alone is about 10% of the control activity.

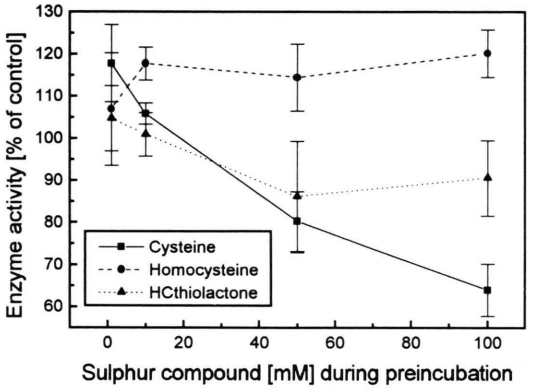

Fig. 6b. Activity of glutamic-pyruvic transaminase (0.1 U) after preincubation (15 min, 37 °C) with various amounts of cysteine, homocysteine or homocysteinethiolactone: 1; 10; 50; 100 mM (without Cu^{2+}).

3) Oxidation of the sulfhydryl groups of cysteine and homocysteine

As shown in Fig. 2 transitionmetal-dependent ethene release from Met in the presence of hydrogenperoxide is inhibited by increasing concentrations of PyP.

During the interaction between copper (Fig. 7) or iron (data not shown) and Cys or HC, the sulfhydryl group of Cys is rapidly oxidized while the one of HC is much more stable (Preibisch and Elstner, 1994). This is probably due to the formation of a relatively stable octohedral HC–Cu

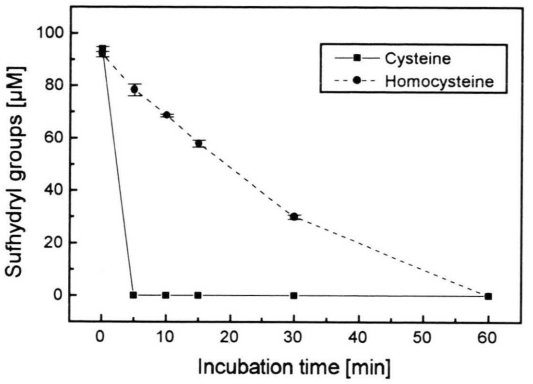

Fig. 7. Reactivities of the sulfhydryl groups of cysteine and homocysteine (100 μM) with Cu^{2+} (5 μM), pH 7.4, 37 °C.

complex as compared to the unstable Cys–Cu complex. This assumption is deduced from semiempirical computer based ZINDO-calculations (Kieninger and Elstner, manuscript in preparation). This hypothesis may explain some of the differences observed in the pro- and antioxidative activities of Cys and HC.

4) Reactions with dihydroxyphenylalanine

The mentioned difference in reactivity between Cys and HC is also observed during the polyphenoloxidase-catalyzed oxidation of the neurotransmitter precurser dihydroxy-phenylalanine (DOPA). It is well known that PPO-catalyzed *o*-diphenol oxidation in the presence of Cys yields cysteinyl-DOPA as a Michael-type addition product of Cys onto the catalysis-derived *o*-quinone. HPLC-analysis of two mayor products (peaks 2a and 2b) of this condensations show that the Cys-derived products represent rapidly produced intermediates which slowly disappear after 60 min reaction time (Fig. 8a) while the HC-derived products seem to accumulate rather slowly (Fig. 8b).

5) Oxidation of low density lipoprotein (LDL)

LDL oxidation can be followed by several methods, such as dieneconjugation, electrophoretic mobility or HPLC-analysis of the composition of the fatty acid contents (Puhl *et al.*, 1994).

Dieneconjugation as an indicator of peroxidizing unsaturated fatty acids in LDL can be photometrically observed starting ca. 30 min after

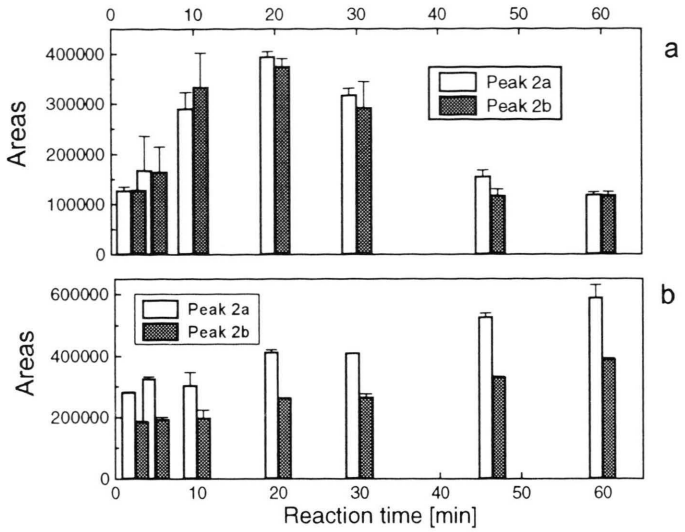

Fig. 8. Separation of the cysteinyl-DOPAquinones (a) and of the homocysteinyl-DOPAquinones (b) by HPLC after 2, 5, 10, 20, 30, 45, 60 min reaction time of polyphenoloxidase (783 U) with DOPA (9.6 mM) in the presence of cysteine or homocysteine (0.8 mM), pH 6.0, room temperature.

incubation with 2.5 μM or 5 μM Cu^{2+}. Copper concentrations below 1 μM show no effect and 10 μM catalyze identical peroxidation as 5 μM. 500 μM Cys or HC completely inhibit this process (Fig. 9).

Changes in the electrophoretic mobility of LDL on 0.8% agarose gel may represent a modification of the Apo B-100 moiety. We incubated LDL in the presence of 5 μM Cu^{2+} with various concentrations of Cys, HC or HCTL ranging from 50 to 500 μM for 24 hours at 37 °C. Results are presented as *R*f-values (relative mobility) in Table I.

Treatment of LDL with 5 μM Cu^{2+} clearly increases its electrophoretic mobility while Cys annihilates the Cu^{2+}- effect in a concentration-dependent manner. The copper-increased mobility is further augmented by HC. HCTL also enhances the copper effect, but no clear correlation between concentration and changes in electrophoretic mobility can be observed.

Table I. *R*f-values (agarose gel) of LDL preincubated with Cu^{2+} 5 μM and several test substances. The relative mobility of LDL incubated with Cu^{2+} 5 μM alone is set as 1.00; the *R*f-value for untreated LDL is 0.89.

Test substance	50 μM	100 μM	250 μM	500 μM
Cys	1.00	0.92	0.91	0.89
HC	1.09	1.14	1.14	1.17
HCTL	1.17	1.17	1.06	1.14

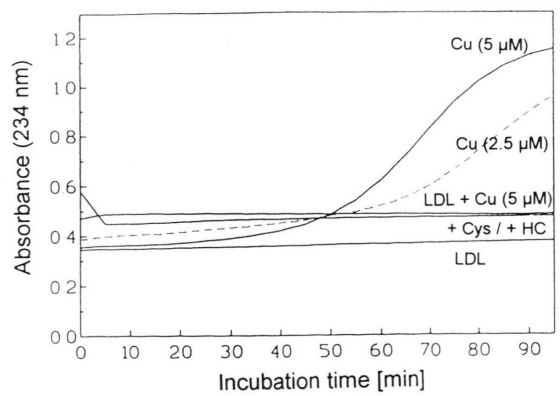

Fig. 9. Monitoring of dieneconjugation in low-density lipoprotein caused by incubation with Cu^{2+} (various amounts), cysteine and homocysteine (500 μM) by continuous recording changes in absorbance at 234 nm.

Discussion

The transitionmetal ions copper and iron together with HC are under discussion in oxidatively causing neurodegenerative and arteriosclerotic diseases. Decreases or imbalances of the vitamins B_6, B_{12} and folic acid seem to be cooperatively or causally involved in several categories of the above disease fields (Schäfer-Elinder and Waldius, 1994; Santhosh-Kumar *et al.*, 1994; Bell *et al.*, 1992).

The transamination product of Met, α-ketomethylthiobutyrate (KMB), is one product of Met metabolism especially in persons with inborn Met adenosyltransferase deficiency (Blom *et al.*, 1989).

It reacts with a wide spectrum of oxidants producing ethene, methanethiol and other products. As reported previously (Preibisch and Elstner, 1994), homocysteine in the presence of either copper- or iron-ions stimulates ethene release from KMB in a concentration-dependent manner. In the presence of either 5 μM Fe^{2+}, 5 μM Fe^{3+} or 25 μM Cu^{2+} ions KMB is fragmented yielding ethene. The effects of homocysteine or its thiolactone are enhanced by Fe^{3+} and Cu^{2+}. The stimulating effects of cysteine and homocysteinethiolactone are completely abolished in the presence of catalase while superoxide dismutase has less effect. The chain breaking radical scavenger propylgallate strongly inhibits the thiolactone effect but has only little effect on the cysteine system. Since the hydrogenperoxide forming capacity is much stronger with cysteine as compared to homocysteine or its thiolactone the rapid formation of a free radical in the thiolactone ring after reacting with Cu^{2+} seems to represent the critical step in the destructive capacity of the thiolactone. The following sequence of events might be operating, where $HC^{\cdot+}$ or $HC(TL)^{\cdot+}$ may represent a HC- or HCTL-thiylkation radical, i.e. a HC- or HCTL-molecule with an unpaired electron at the sulphur atom:

a) $2\ HC(TL) + 2\ Cu^{2+} \rightarrow 2\ HC(TL)^{\cdot+} + 2\ Cu^{1+}$

b) $2\ Cu^{1+} + 2\ O_2 \rightarrow 2\ Cu^{2+} + 2\ O_2^{\cdot-}$

c) $2\ O_2^{\cdot-} + 2\ H^+ \rightarrow H_2O_2 + O_2$

d) $H_2O_2 + Cu^{1+} \rightarrow Cu^{2+} + OH^- + OH^{\cdot}$

e) $OH^{\cdot}$ + KMB → ethene + other products

Reaction a) results in the production of excitatory neurotransmitters (sulfinic acids, cysteic and homocysteic acids) on the one hand and inhibitory transmitters (taurine, hypotaurine) on the other hand (Santhosh-Kumar *et al.*, 1994) via the reaction as ligands for the NMDA-(N-methyl-D-aspartate-) type of glutamate receptors. HC-induced elevated extracellular (dialyzable) aspartate and taurine levels and reduced GABA (γ-amino butyric acid) levels suggesting the creation of an imbalance in these neurotransmitters in the hippocampus have recently been reported by Butcher *et al.*,1991.

In this context it is interesting to note that oxidativ "stress" releases excitatory amino acids (L-glutamate and D-aspartate) by cerebral cortical synaptosomes and oxidation of Cys- or Met-residues in proteins produces a decrease in synaptic potentials in the hippocampus (Gilman *et al.*, 1993 and refs. therein). A very similar type of reaction is known to inactivate protease inhibitors via Met-oxidation through the cooperative reaction of iron-thiolate with endogenous histidine residues (Schöneich *et al.*, 1993) according to the above reaction sequence a) to d).

Reaction d) represents a so-called "Fenton-Type" of strong oxidant production from hydrogenperoxide catalyzed be either copper- or iron-ions, reoxidizing the metal catalyst for reaction a). These oxidants, similar to the $OH^{\cdot}$ radical react extremely rapidly with KMB and slower with Met producing ethene. As shown in Fig. 2, PyP is a strong inhibitor of the copper system and is degraded during its reaction (data not shown). This protective role is also expressed during the copper-catalyzed and Cys- or HCTL-enhanced inactivation of transaminases (TA), where characteristic differences between glutamic-oxaloacetic TA and glutamic-pyruvic TA are observed. The latter enzyme seems to be especially sensitive to copper ions.

PyP is also rapidly consumed forming a new product in the presence of thiols such as Cys or HC. PyP deficiency in certcain cases, especially in elderly people frequently exhibiting elevated HC levels (Bell *et al.*, 1992) may thus be explainable not only by nutritional shortcomings but also by derivatization or cooxidation with HC. Similarly, the concentrations and relative distribution of aromatic amines such as DOPA, dopamine, 6-hydroxyDOPA and noradrenalin are critical in age-associated memory impairment, dementia of depressed elderly people as well as Parkinson's and Alzheimer's diseases (Bell *et al.*, 1992; Gottfries, 1985; McEntee and Crook, 1990; Kopin, 1993). As shown in Figs 8a and 8b DOPA reacts with both Cys and HC. The products are different both in quality and in quantity where the products with Cys seem to represent rapidly formed transitory states while the ones with HC accumulate slowly. Independent of the monoamine oxidase-catalyzed, the macrophage-initiated and transition metal-catalyzed autoxidation of these neurotransmitters (Youdim and Lavie, 1994) the derivatization of the *o*-dihydroxy aromats by HC may represent an-

other pathway for the induction of neurodegenerative processes.

Atherogenic processes seem to be initiated or augmented by oxidative modification of LDL (Schäfer-Elinder and Waldius, 1994; Ross, 1993) where structural configuration as well as the chemical composition of the macromolecule determines the oxidation resistance. Especially a dense LDL fraction (Dejager *et al.*, 1993) and LDL species with low sialic acid contents (Tertow *et al.*, 1993) seem to exhibit diminished oxidation resistance (Ross, 1993) and/or accumulate certain atherogenic lipids such as cholesterol (Tertow *et al.*, 1993). We tested two different LDL preparations from different donors as to their optical (diene-conjugation, Fig. 9) and chromatographic (gel electrophoresis, Table I) behaviours after oxidative treatment with copper ions in the presence or absence of HC. The LDL preparation from one donor exhibited the "normal" copper effect but no enhancement by HC (data not shown). The LDL from the second donor showed a reproducible overadditive HC-copper effect in the electrophoretic test and a complete inhibition of the diene-conjugation by Cys or HC. This inhibitory effects are in contrast to the findings by Lupo *et al.* (Lupo *et al.*, 1994). The difference may be explaind by the extremely different concentrations of copper and HC described by Kopin, 1993 and/or differences in the two experimental approaches. Our findings are in perfect agreement with the results recently published by Hirano *et al.*, 1994 who showed an HC-induced, iron-catalyzed LDL oxidation which was prevented by α-tocopherol.

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