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Homocysteine promotes the LDL oxidase activity of ceruloplasmin

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Abstract Ceruloplasmin (CP) oxidises low density lipoprotein (LDL). The oxidising potential depends on the formation of Cu^{2+}-CP which is redox-cycled during oxidation. Homocysteine (HCY) reduces free Cu^{2+}, potentiating its cell-damaging property. We show that HCY enhanced LDL oxidation by CP, but did not activate the LDL oxidising potential of Cu^{2+}-diamine oxidase. Selective removal of the redox-active Cu^{2+} abolished the LDL oxidase activity of CP. However, HCY partially restored the LDL oxidase activity of redox-copper depleted CP, indicating that the remaining six copper atoms in CP may also be involved in the process. Spectroscopic and oxidation inhibition studies using the Cu^{+}-reagent bathocuproine revealed that HCY induced Cu^{+}-CP formation, thus promoting its LDL oxidase activity.

Key words: Homocysteine; Ceruloplasmin; Copper ion; LDL oxidation

1. Introduction

The oxidative modification of low density lipoprotein (LDL) may play an important role in the early events of atherogenesis [1–4]. The in vivo pathophysiologically relevant pathway(s) of atherogenic LDL alterations, however, have not been elucidated at present. In vitro studies have shown that LDL can be altered by a variety of biochemical reactions, e.g. modification by endothelial cells, smooth muscle cells, peroxynitrite, peroxidases, metmyoglobin, copper ions, NO radicals, tocopheryl radicals and ceruloplasmin (CP) [5–17]. LDL oxidation by free copper ions is used as a convenient system to study anti- and pro-oxidant compounds. In this respect the observation that the copper transport protein CP could stimulate the oxidation of LDL by vascular cells in vitro has brought an additional aspect of copper-dependent LDL oxidation [18–21]. The generation of Cu^{+} in CP has been proposed as a prerequisite of its LDL oxidising potential [22]. Recently it has been reported that homocysteine (HCY) can reduce Cu^{2+} to Cu^{+} [23] and this reaction could potentiate the cell-damaging property of copper ions to endothelial and neuronal cells in presence of HCY [23,24]. As HCY is an independent risk factor for atherosclerosis [25] and total plasma HCY and copper correlate in patients with peripheral vascular disease [26] we have tested the influence of HCY on the LDL oxidising potential of CP. The results show that HCY could enhance the oxidation of LDL by CP via the formation of Cu^{+}-CP.

2. Materials and methods

CP (human) was from Calbiochem and had a purity of >95% as analysed by SDS–PAGE. D,L-Homocysteine, D,L-homocystine, D,L-cysteine, D,L-methionine, bathocuproine disulphonate and diamine oxidase (DAO; EC 1.4.3.6, from porcine kidney, 0.07 U/mg protein) were from Sigma-Aldrich Chemical Corp. Chelex-100 was supplied by Bio-Rad. All other chemicals used were of analytical grade.

2.1. Lipoprotein isolation

LDL and HDL preparations were isolated by ultracentrifugation as reported previously [27]. The final preparations were dialysed against 150 mmol/l NaCl containing 0.1 mmol/l ethylenediamine tetraacetic acid and filter-sterilised. Protein was estimated by [28] using bovine serum albumin as a standard. All LDL concentrations are given as mg protein/ml. Results were obtained with three different LDL preparations.

2.2. LDL oxidation

Prior to LDL oxidation, the lipoprotein was equilibrated in phosphate buffered saline (PBS) pH 7.4 using Sephadex G-25 chromatography (PD-10 columns, Pharmacia). LDL (0.2 mg/ml) was incubated with the respective compound at 37°C for 4 h.

2.3. CP preparation

Lyophilised CP was dissolved in distilled water and applied to a small Sephadex column (Nick-column, Pharmacia) equilibrated in PBS and the protein fraction was used in the experiments.

2.4. Estimation of LDL oxidation

Thiobarbituric acid reactive substances (TBARS) were estimated according to [29] extending the reaction time to 45 min. A molar extinction coefficient of 156 000 mol/l/cm was used to calculate malonaldehyde formed.

2.5. Lipoprotein electrophoresis

Aliquots (10 μl) of treated or untreated LDL were applied to agarose gels (1% in veronal buffer) and run for 90 min and lipoproteins were detected according to the supplier of the analytical system (Lipidophor All In, Immuno-Baxter AG). Measurement of relative electrophoretic mobility (REM) was taken as an indicator of LDL oxidation [30], setting the electrophoretic mobility of native (untreated) LDL arbitrarily as 1.

2.6. Removal of redox-active copper

Redox-active copper from CP was removed according to [31] using Chelex-100 treatment of CP in PBS for 4 h at 23°C.

2.7. Spectroscopy

Free Cu^{2+} (50 μmol/l in PBS) were incubated with 100 μmol/l bathocuproine disulphonate (BCD) in the absence or presence of 10 μmol/l of the respective thio-amino acid for 1 h at 37°C and subsequently the

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spectra were recorded using a Hitachi UV/VIS 2000 spectrophotometer.

CP (1.66 μmol/l in PBS) was incubated in the absence or presence of 100 μmol/l thio-amino acids and 100 μmol/l BCD for 1 h at 37°C. Subsequently spectra were recorded.

To avoid interactions of free Cu^{2+} with PBS, Cu^{2+}–glycine complexes (molar ratio 1:6) were used according to [23].

2.8. Estimation of copper

Copper content of DAO was estimated by atomic absorption in an aliquot of a DAO solution (10 mg/ml in 0.15 mol/l NaCl) using a Perkin Elmer 5100 ZL atomic absorption spectrometer.

3. Results

3.1. Time course of copper ion-mediated LDL oxidation in presence of HCY

Time course experiments with incubations up to 21.5 h revealed that maximal stimulation of free Cu^{2+} (0.2 μmol/l) mediated LDL oxidation by HCY (100 μmol/l) was found between 2 and 4 h of incubation, as can be seen in Fig. 1. Therefore all subsequent experiments were done with a standard incubation time of 4 h.

3.2. Influence of different HCY concentrations on LDL oxidation induced by CP

As seen in Fig. 2, HCY up to 100 μmol/l stimulated LDL oxidation in presence of CP (25 μg/ml) and Cu^{2+}, as indicated by increased malondialdehyde formation and alteration of the apoprotein (see inset Fig. 2). In contrast, the LDL oxidising potential of DAO, a copper enzyme [32] which has been shown to stimulate endothelial cell-mediated LDL oxidation [33], was not activated by HCY.

3.3. Influence of HCY, homocystine (HCYin), cysteine (Cyst) and methionine (Meth) on CP-induced LDL oxidation

From the biological thio-amino acids tested, HCY was the only one which stimulated CP-mediated LDL oxidation. Cyst, HCYin, and Meth showed some inhibitory action (Table 1). HCY pretreatment of LDL (2 h at 37°C, subsequent removal of HCY) did not increase the susceptibility of LDL to CP-mediated oxidation (data not shown).

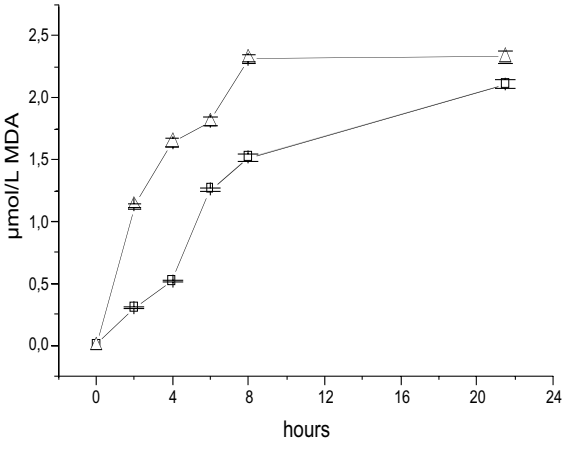

Fig. 1. Time course of copper ion-mediated LDL oxidation in presence of HCY. LDL (0.2 mg/ml) was incubated at 37°C in the absence or presence of HCY (100 μmol/l) and 0.2 μmol/l Cu^{2+}. Malondialdehyde was measured ($n=2$) as given in Section 2. □, Cu^{2+}; △, Cu^{2+}+HCY.

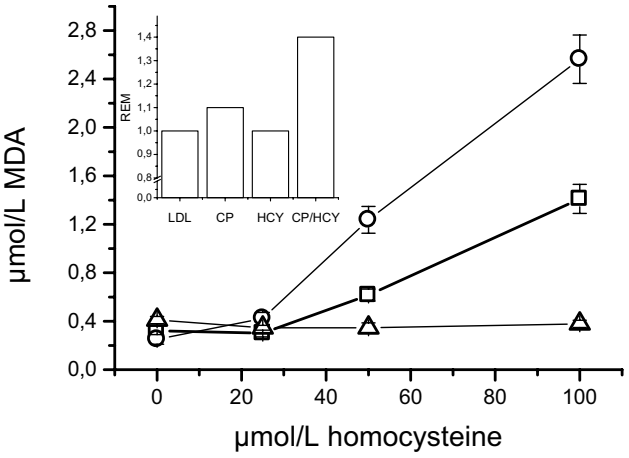

Fig. 2. Influence of HCY on LDL oxidation induced by CP, DAO and copper ions. LDL (0.2 mg/ml) was incubated for 4 h at 37°C in the absence or presence of HCY (up to 100 μmol/l) and the respective inducer (at concentrations equivalent to 0.2 μmol/l Cu^{2+}) of lipid oxidation. Malondialdehyde was measured ($n=2$) as given in Section 2. △, DAO; □, CP; ○, Cu^{2+}. Inset: REM of LDL treated with CP and HCY as given above.

3.4. Effect of the removal of redox-active Cu^{2+} on HCY/CP-induced LDL oxidation

Table 2 shows the effect of copper removal on the HCY-stimulating effect on CP-mediated LDL oxidation. Chelex treatment of CP completely abolished the LDL oxidising property of the protein, in accordance with [31]. Addition of HCY (100 μmol/l) to Chelex-treated CP still stimulated the ability of the preparation to oxidise LDL, although LDL oxidation was less pronounced than with untreated CP.

3.5. CP Cu^{2+} reduction by HCY and effect of bathocuproine disulphonate on HCY/CP-induced LDL oxidation

Cu^{2+} reduction in CP may trigger the protein's LDL oxidase activity [22,31]. As seen in Fig. 3, using the Cu^{+}-reagent bathocuproine disulphonate, HCY and HCYin strongly reduced free Cu^{2+} – indicated by the formation of the coloured Cu^{+} complex (λ max = 475 nm) after 1 h at 37°C. It should be mentioned that HCYin-mediated Cu^{+} formation was considerably slower than that of HCY (data not shown). Cyst was less effective (20–25% of HCY), cystine and Meth had no copper-reducing effect. As seen in the inset to Fig. 3, HCY was also able to reduce Cu^{2+} in CP. If Cu^{+} formation in CP by HCY is a prerequisite of the stimulating effect, bathocuproine should inhibit the HCY effect on CP-mediated LDL oxidation. As seen in Fig. 4, bathocuproine strongly inhibited

Table 1
Influence of HCY, HCYin, Cyst and Meth on CP-induced LDL oxidation

Treatment	% of control
LDL	100
LDL+CP	233
LDL+CP+HCY	728
LDL+CP+HCYin	160
LDL+CP+Meth	105
LDL+CP+Cyst	143

LDL (0.2 mg/ml) was incubated for 4 h at 37°C in the absence or presence of the respective amino acids (100 μmol/l each) and CP (25 μg/ml). Malondialdehyde was measured as given in Section 2. Means of two measurements are given.

Table 2
Effect of the removal of redox-active Cu^{2+} on HCY/CP-induced LDL oxidation

Treatment	% of control
LDL	100
LDL+CP	448
LDL+CP+HCY	1106
LDL+CP chel	112
LDL+CP chel+HCY	438

Redox-active Cu^{2+} was removed from CP as given in Section 2. LDL (0.2 mg/ml) was incubated for 4 h at 37°C in the absence or presence of the respective CP (25 μg/ml) preparation (CP: untreated CP, CP chel: redox-active Cu^{2+} removed). Malondialdehyde was measured as given in Section 2. Means of two measurements are given.

the HCY-promoted LDL oxidation. Bathocuproine was by far less effective in inhibiting the CP-mediated LDL oxidation in absence of HCY.

3.6. Effect of HDL on HCY/CP-induced LDL oxidation

HDL can act as an antagonist of LDL oxidative modification [34,35]. Thus we have tested the influence of HDL on the enhancing effect of HCY on CP-mediated LDL oxidation. As seen in Fig. 5, the CP-mediated LDL oxidation was not affected by increasing concentrations of HDL, whereas, as reported previously [36], the free copper ion-mediated reaction was strongly inhibited. HDL-depressed lipid oxidation in HCY facilitated CP- and Cu^{2+}-mediated LDL oxidation (Fig. 5).

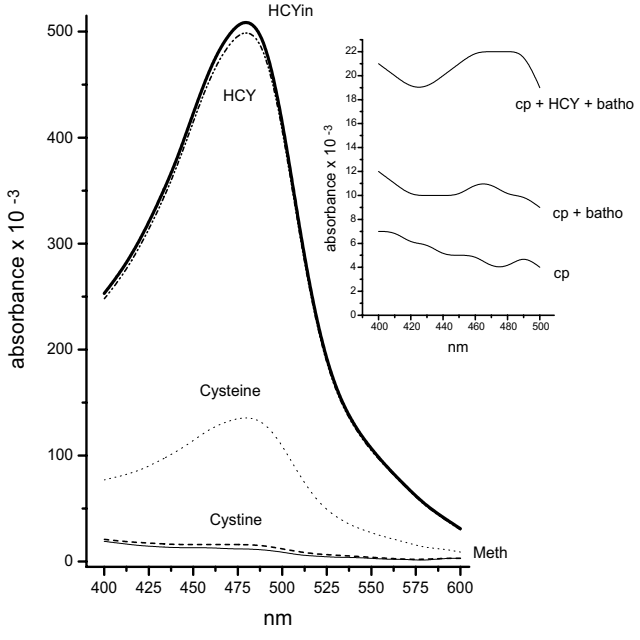

Fig. 3. Cu^{2+}-reducing activity of HCY, HCYin, Cyst, cystine and Meth. Free Cu^{2+} ions (50 μmol/l in PBS) were incubated with 100 μmol/l BCD in the absence or presence of 10 μmol/l of the respective thio-amino acid for 1 h at 37°C and subsequently the spectra were recorded. Inset: Cu^{2+} reduction in CP by HCY. CP (1.66 μmol/l in PBS) was incubated with 100 μmol/l BCD in the absence or presence of 100 μmol/l HCY for 1 h at 37°C. Subsequently spectra were recorded.

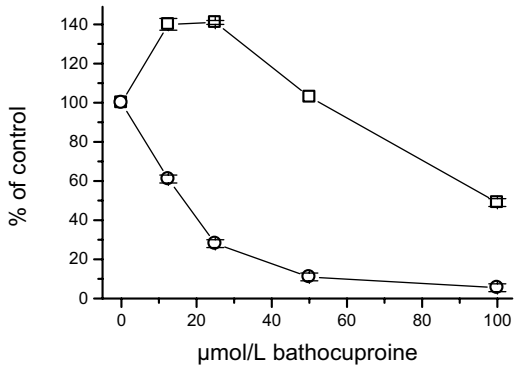

Fig. 4. Effect of bathocuproine disulphonate on HCY/CP-induced LDL oxidation. LDL (0.2 mg/ml) was incubated with CP for 4 h at 37°C in the absence or presence of bathocuproine (100 μmol/l) with or without HCY (100 μmol/l). Malondialdehyde was measured as given in Section 2. □, CP; ○, CP+HCY.

4. Discussion

Reduction of Cu^{2+} to Cu^{+} may play a role in lipid peroxidation. Cu^{2+}-reducing factors like lipid hydroperoxides (Cu^{2+}+LOOH → Cu^{+}+LOO$^{\bullet}$+H^{+}), α-tocopherol (Cu^{2+}+α-Toc-OH → Cu^{+}+α-Toc-O$^{\bullet}$+H^{+}), ascorbic acid (Cu^{2+}+AscH^{-} → Cu^{+}+Asc$^{\bullet-}$+H^{+}) and thiols (Cu^{2+}+RSH → Cu^{+}+1/2 RSSR+H^{+}) have been described. For a recent review on copper-dependent LDL oxidation see Burkitt [37]. Although redox-active copper and iron ions had been found in atherosclerotic plaques [38], the pathophysiological role of transition metal ion-mediated LDL oxidation has been discussed controversially. CP has been found to oxidise LDL in vitro [18–22,31] qualitatively like free copper ions, an observation which brought an in vivo aspect in the transition metal ion-mediated LDL oxidation, although some authors have raised critical points regarding CP-mediated lipid peroxidation due to the extremely labile nature of this plasma protein [39,40]. Formation of Cu^{+}-CP by superoxide radicals may trigger the LDL oxidation potential of the protein in cell-mediated LDL oxidation systems [21]. In this respect, Feichtenhofer et al. [41] have recently shown that ascorbate/dehydroascorbate could activate CP as an LDL oxidase presumably by reducing the

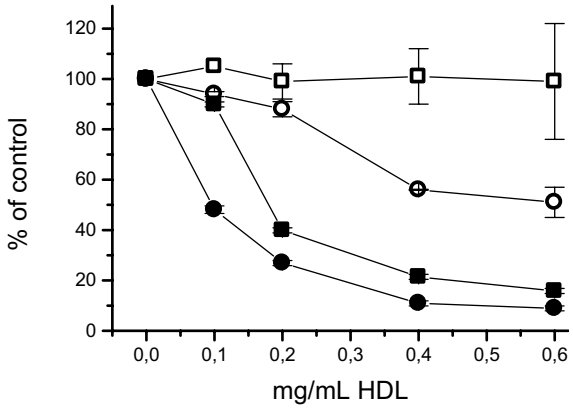

Fig. 5. Effect of HDL on HCY/CP-induced LDL oxidation. LDL (0.2 mg/ml) was incubated for 4 h at 37°C in the absence or presence of HCY (up to 100 μmol/l) and CP or free Cu^{2+}. The effect of HDL (up to 0.6 mg/ml) on LDL oxidation was studied. □, CP; ○, CP+HCY; ■, free Cu^{2+}; ●, free Cu^{2+}+HCY. Malondialdehyde was measured ($n = 2$) as given in Section 2.

redox-active Cu^{2+} in CP. HCY has been shown to reduce free Cu^{2+} [23]. Thus one may assume that HCY may activate the LDL oxidase property of CP by reducing the redox-active Cu^{2+} of the protein. HCY up to 100 μmol/l stimulated LDL oxidation in presence of CP. In contrast, the LDL oxidising potential of Cu^{2+} DAO was not activated by HCY. This may be due to the location of the copper atom in the protein, which – in contrast to CP, where the redox-active copper is located near the surface of the protein [22] – may make the access of the activator more difficult. The oxidation potential of equivalent amounts of free copper ions (0.2 μmol/l) was also enhanced by HCY under these conditions. Lynch et al. [42] reported no effect of HCY on free Cu^{2+}-mediated LDL oxidation as assessed by agarose gel electrophoresis. These authors used 16 h incubations (10 μM Cu^{2+}/1 mM HCY) instead of 4 h as used in the present study. A time course experiment showed that maximal stimulation of free Cu^{2+} (0.2 μmol/l) mediated LDL oxidation by HCY (100 μmol/l) was found between 2 and 4 h of incubation. In accordance with the observation of Lynch, no stimulation was found when the incubation time was prolonged up to 21.5 h.

From the biological thio-amino acids tested, HCY was the only one which stimulated CP-mediated LDL oxidation. Cyst, HCYin, and Meth where ineffective. HCY pretreatment of LDL did not increase the susceptibility of LDL to CP-mediated oxidation (data not shown). By the removal of one of the seven copper atoms in CP – the redox-active one – by Chelex treatment it has been shown that this preparation was unable to stimulate in vitro LDL oxidation [31]. In accordance with this report Chelex treatment completely abolished the LDL oxidising property of CP in our system. However, addition of HCY to Chelex-treated CP still stimulated the ability of the preparation to oxidise LDL, although not as strongly as with the untreated CP. Thus, the remaining six copper atoms in CP may be partially involved in the HCY effect on LDL oxidation by CP. This could be due to mobilisation of redox-active copper ions from CP by HCY resulting in facilitated lipid oxidation, a mechanism which has been supposed for the peroxynitrite-promoted plasma lipoprotein oxidation mediated by CP [40]. Cu^{2+} reduction in CP may trigger the protein's LDL oxidase activity [22,31]. HCY was able to reduce CP-bound Cu^{2+} (and free Cu^{2+}) as spectroscopically analysed by the formation of the Cu^{+}–bathocuproine complex. Addition of bathocuproine to the CP/HCY/LDL system resulted in strong inhibition of lipid oxidation. In contrast, bathocuproine was less effective in inhibiting the CP-mediated LDL oxidation in absence of HCY. Thus, Cu^{2+} reduction in CP by HCY may be a prerequisite of the stimulating effect. The exact reason for the increase in CP-mediated LDL oxidation found at low bathocuproine concentrations is not known. In this respect Pinchuk et al. [43] reported that the Cu^{+} chelators neocuproine and bathocuproine at certain chelator/copper ratios could even stimulate LDL oxidation. Although HCYin was able to reduce Cu^{2+} to the same extent as HCY, HCYin did not stimulate the CP-mediated LDL oxidation. This could be due to the molar ratio of copper ions/thio-amino acids, as has been reported by Heinecke et al. [44] for the influence of e.g. glutathione and HCY on Cu^{2+}-mediated LDL oxidation. HDL can act as an antagonist of LDL-oxidative modification [34,35]. In contrast to free copper ions HDL did not affect the CP-mediated LDL oxidation. HDL has been shown to lose its property to protect LDL against oxidative modification by endothelial cells in presence of CP [45,46], presumably by binding of CP to HDL and inactivating associated paraoxonase. The stimulating effect of HCY on lipid oxidation was counteracted by HDL supporting HDL as an antiatherogenic factor [34,35].

The results show that HCY facilitates the lipid-oxidising potential of CP. Reduction and release of Cu^{2+} in CP may be a mechanism by which HCY stimulates LDL oxidation by this acute phase protein.

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