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Copper- and magnesium protoporphyrin complexes inhibit oxidative modification of LDL induced by hemin, transition metal ions and tyrosyl radicals

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Abstract

The oxidative modification of LDL may play an important role in the early events of atherogenesis. Thus the identification of antioxidative compounds may be of therapeutic and prophylactic importance regarding cardiovascular disease. Copper-chlorophyllin (Cu-CHL), a Cu^{2+}-protoporphyrin IX complex, has been reported to inhibit lipid oxidation in biological membranes and liposomes. Hemin (Fe^{3+}-protoporphyrin IX) has been shown to bind to LDL thereby inducing lipid peroxidation. As Cu-CHL has a similar structure as hemin, one may assume that Cu-CHL may compete with the hemin action on LDL. Therefore, in the present study Cu-CHL and the related compound magnesium-chlorophyllin (Mg-CHL) were examined in their ability to inhibit LDL oxidation initiated by hemin and other LDL oxidizing systems. LDL oxidation by hemin in presence of H_2O_2 was strongly inhibited by both CHLs. Both chlorophyllins were also capable of effectively inhibiting LDL oxidation initiated by transition metal ions (Cu^{2+}), human umbilical vein endothelial cells (HUVEC) and tyrosyl radicals generated by myeloperoxidase (MPO) in presence of H_2O_2 and tyrosine. Cu- and Mg-CHL showed radical scavenging ability as demonstrated by the diphenylpicrylhydracylradical (DPPH)-radical assay and estimation of phenoxyl radical generated diphenyl (dityrosine) formation. As assessed by ultracentrifugation the chlorophyllins were found to bind to LDL (and HDL) in serum. The present study shows that copper chlorophyllin (Cu-CHL) and its magnesium analog could act as potent antagonists of atherogenic LDL modification induced by various oxidative stimuli. As inhibitory effects of the CHLs were found at concentrations as low as 1 μmol/l, which can be achieved in humans, the results may be physiologically/therapeutically relevant.

Keywords: *LDL oxidation, hemin, antioxidant, protoporphyrin, chlorophyllin, atherosclerosis*

Abbreviations: *Cu-CHL, copper-chlorophyllin; Mg-CHL, magnesium-chlorophyllin; HUVEC, human umbilical vein endothelial cells; DPPH, diphenylpicrylhydracylradical; MPO, myeloperoxidase; TBARS, thiobarbituric reactive substances; TF, tissue factor*

Introduction

The oxidative modification of LDL particles may play a pivotal role in early stage atherogenesis [1–4]. This observation has led to studies focusing on the mechanisms of LDL oxidation and on the antioxidant potential of drugs or naturally occurring compounds. *In vitro*, LDL oxidation can be induced by transition metal ions (Fe, Cu), reagent hypochlorite, superoxide/nitric oxide, azocompounds,

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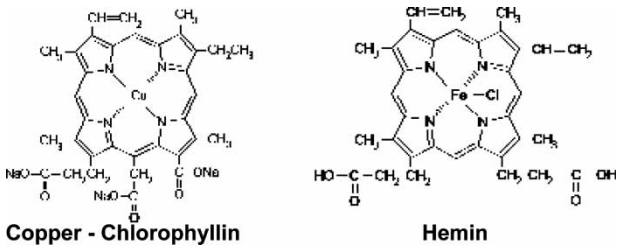

Scheme 1.

vascular cells and peroxidases [5–14]. In addition to myeloperoxidase (MPO), an enzyme secreted by activated phagocytes, which can generate tyrosyl and NO_2 radicals [15–17], hemoglobin and hemin have also been identified as possible candidates of *in vivo* LDL alterations. Hemin is a Fe^{3+}-protoporphyrine IX complex, which binds to LDL and induces LDL oxidation in presence of H_2O_2 or lipid hydroperoxides present in LDL [18–26]. The structurally related Cu^{2+}-protoporphyrine IX complex, copper chlorophyllin (Cu-CHL) has been found to have antioxidant potential, as studied in liposomes and isolated mitochondrial membrane systems [27,28] exposed to gamma radiation. Assuming that chlorophyllin may compete with the hemin/LDL interaction due to its similar chemical structure (see Scheme 1), we have tested Cu-CHL and its parent compound magnesium-chlorophyllin (Mg-CHL) in their ability to inhibit LDL oxidation induced by hemin. The influence of both chlorophyllins on other established LDL oxidizing systems like transition metal ion dependent (Cu^{2+}), endothelial cell and tyrosyl radical mediated LDL oxidation were also studied.

Materials and methods

Hemin, Copper-chlorophyllin (Cu-CHL), diphenylpicrylhydracylradical (DPPH), ebselen (2-Phenyl-1,2-benzisoselenazol-3(2H)-one), pyrogallol, tyrosine-sodium salt were from Sigma Chemicals. Mg-CHL was a generous gift of Paninkret, Germany. Myeloperoxidase (MPO, EC 1.11.17, purity >95% as assessed by SDS gel electrophoresis) was purchased from Calbiochem-Novabiochem International. Hemin was dissolved in 20 mmol/l NaOH and further diluted in phosphate buffered saline (PBS) pH 7.4. Daily hemin and chlorophyllin solutions were prepared fresh and kept under light protection until use.

Lipoprotein isolation

LDL and HDL were isolated by ultracentrifugation as reported previously [29]. The final preparations were dialyzed against 150 mmol/l NaCl containing 0.1 mmol/l EDTA and filter sterilized. Protein was estimated by [30] using bovine serum albumin as a standard. All LDL and HDL concentrations are given as mg protein/ml.

LDL oxidation

Prior to LDL oxidation, the lipoprotein was equilibrated in phosphate buffered saline pH 7.4 (PBS) using Sephadex G-25 chromatography (PD-10 columns, Pharmacia).

Hemin induced oxidation

LDL (0.2 mg/ml PBS) was incubated in the presence of 2.5 μmol/l hemin and 40 μmol/l H_2O_2 at 37°C for the indicated time.

Copper ion induced oxidation

LDL (0.2 mg/ml PBS) was incubated in the presence of 5 μmol/l Cu^{2+} at 37°C for the indicated time.

Endothelial cell mediated oxidation

Human umbilical vein endothelial cells (HUVEC) were isolated, cultured and used for cell mediated LDL oxidation as reported previously [31,32]. LDL concentration was 0.1 mg/ml RPMI medium and oxidation time was 18 h.

Tyrosyl radical (MPO) mediated oxidation

LDL (0.2 mg/ml) in 0.05 mol/l phosphate buffer pH 7.5 containing 0.1 mmol/l DTPA and MPO (3 nmol/l) was incubated in the presence of H_2O_2 (40 μmol/l) and tyrosine (50 μmol/l) as substrate [15] at 37°C for the indicated time.

Estimation of lipid oxidation

Conjugated diene. Lipid oxidation in LDL was analyzed by monitoring conjugated diene formation as the increase in absorbance at 234 nm ($\varepsilon = 2.95 \times 10^4\,M^{-1}\,cm^{-1}$) [33] using a Hitachi U-2001 spectrophotometer with thermostated 6-cell cuvette positioner.

Malondialdehyde formation. Malondialdehyde formation was estimated as reported recently [34] using $\varepsilon = 15.6 \times 10^4\,M^{-1}\,cm^{-1}$ for calculation and were expressed as TBARs equivalents.

Lipid hydroperoxides. Lipid hydroperoxides were estimated as reported previously using the CHOD iodine reagent [35].

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Binding of hemin and chlorophyllins to lipoproteins in serum

Serum was diluted in PBS 1:5 and incubated with hemin or the respective chlorophyllin (all 125 μmol/l) in the presence of 10 μmol/l BHT for 15 min at room temperature. (2 ml total volume). Subsequently the samples were subjected to KBr gradient ultracentrifugation [18]. Cholesterol in the fractions (0.4 ml) was estimated by a commercially available automated method on an Integra 700 analyzer (Roche Diagnostics) and distribution of the respective Cu^{2+}, Fe^{3+} and Mg^{2+}-porphyrines by monitoring the absorbance at 405 nm. Moreover, binding of the colored compounds to LDL and HDL was documented photographically. In separate experiments whole serum was incubated with the respective chlorophyllin (125 μmol/l) and subjected to KBr gradient ultracentrifugation and the LDL fractions isolated. Subsequently KBr was removed by gel filtration and the isolated lipoprotein subjected to lipid oxidation as indicated in the figure legends.

Binding of hemin and chlorophyllins to isolated lipoproteins

LDL or HDL (both 0.5 mg/ml) was incubated in the absence or presence of hemin, Cu-CHL or Mg-CHL (100 or 50 μmol/l) for 30 min at room temperature. 20 μmol/l BHT and 20 μmol/l EDTA was added to avoid lipoprotein oxidation during incubation. Subsequently 15 μg of the respective lipoprotein was subjected to agarose gel electrophoresis (100 V, 30 min). Lipoproteins were stained with Coomassie blue. Binding of the compounds to the lipoproteins was indicated by the alteration of the relative electrophoretic mobility (REM) [18].

DPPH radical scavenging assay

Radical scavenging ability of the chlorophyllins was estimated following the procedure as published previously [36] using pyrogallol as a positive radical scavenging control.

Phenoxyl radical-mediated diphenyl (dityrosine) formation

Diphenyl formation was estimated spectroscopically as previously reported [37]. In brief, tyrosine (1 mmol/l) in 50 mmol/l phosphate buffer containing 100 μmol/l DTPA pH 7.5 was incubated with 100 μmol/l H_2O_2 and 10 nmol/l MPO at 25°C for 1 h. Spectra were recorded between 400 and 280 nm. Radical induced diphenyl (dityrosine) formation was indicated as the increase in absorbance at 320 nm [38].

Octanol/water partition of chlorophyllins

A measure of 1 ml of Cu- or Mg-CHL (50 μmol/l PBS) was extracted with 1 ml octanol at 25°C by vortexing for 15 s and phases were separated by centrifugation at 3000 rpm for 10 min. The concentration of the respective CHL was measured spectrophotometrically (at their absorbance maxima) in the water phase before and after extraction.

Tissue factor activity assay

Tissue factor (TF) assay for the quantification of the procoagulant activity of the endothelial cells was performed as previously described [31]. After incubations, cells were scrape-harvested and washed three times with PBS. Cells suspended in 500 μl PBS were then sonicated by a cell disruptor (Labsonic U, B. Braun Biotech International) for 15 s at 4°C. The cell lysate was then assayed in a one stage clotting assay for procoagulant activity: 50 μl citrated normal donor platelet-poor plasma were incubated for 1 min with 50 μl of cell lysate at 37°C in prewarmed plastic tubes of a ST-4 coagulometer (Stago); 50 μl $CaCl_2$ (30 mmol/l) were then added and the coagulation time was measured. Control experiments were performed with factor VII- (Sigma), IX- (Technoclone), and X (Biopool) -deficient plasmas to characterize the procoagulant activity measured as TF activity.

Statistical analysis

Data were calculated as means ± standard deviation (SD) of 2 to 5 experiments. Specific effects were evaluated by one-way analysis of variance (ANOVA) plus Tukey-Kramer Multiple Comparisons Test. $p < 0.05$ was regarded statistically significant.

Results

Cu-or Mg-CHL tested at the highest concentration (5 μmol/l) did not induce any lipid oxidation in the presence of H_2O_2 (not shown). Figure 1A and B depict the kinetics of LDL oxidation induced by hemin in presence of H_2O_2. A rapid increase in conjugated diene formation was observed. When LDL was pre-incubated with CHLs for 10 min and subsequently the oxidation reaction was initiated by hemin/H_2O_2 the highest Cu-CHL concentrations (5 and 2.5 μmol/l) exerted a highly significant ($p < 0.001$) inhibitory action on hemin mediated lipoprotein oxidation over the entire time measured. A measure of 1.25 μmol/l led to a highly significant ($p < 0.001$) inhibition up to 45 min. As low as 0.625 μmol/l Cu-CHL still significantly inhibited LDL oxidation up to 30 min ($p < 0.05$) (Figure 1A). At this concentration, lag time increased 20-fold compared to control. Mg-CHL exerted highly

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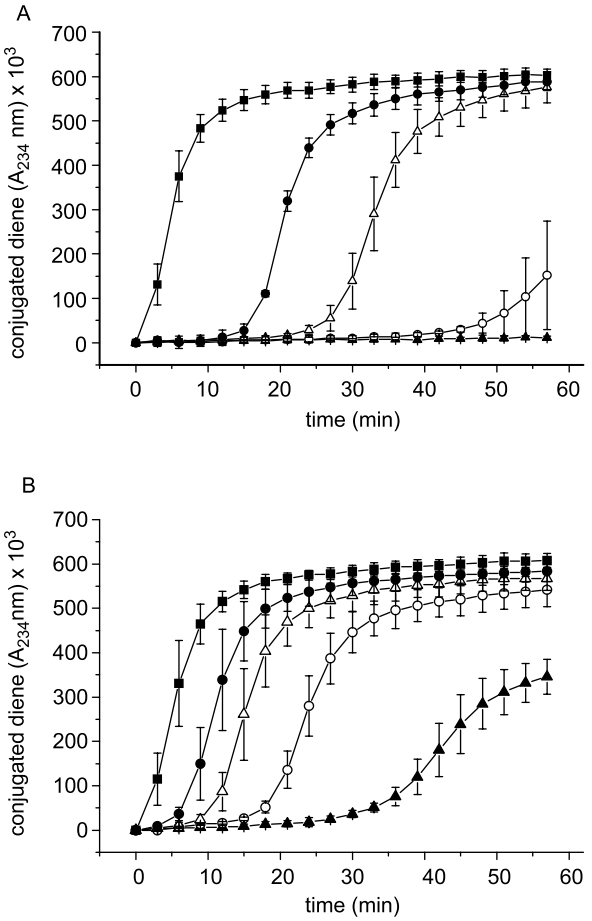

Figure 1. Influence of copper- (A) or magnesium- (B) chlorophyllin on hemin induced LDL oxidation. LDL (0.2 mg/ml PBS) was incubated in the absence or presence of Cu- or Mg-CHL and 2.5 μmol/l hemin and 40 μmol/l H_2O_2 at 37°C and lipid oxidation was measured as the increase in conjugated diene formation as given under "Materials and methods" section. Control:■; 5 μmol/l CHL:▲; 2.5 μmol/l CHL:○; 1.25 μmol/l CHL:△; 0.625 μmol/l CHL:•. Means ± SD are given ($n = 5$).

significant ($p < 0.001$) inhibition at 5 μmol/l over the whole time range. At the end of the incubation period, still significant ($p < 0.05$) inhibition at 2.5 μmol/l was found. A measure of 1.25 and 0.625 μmol/l significantly inhibited lipid oxidation up to 24 and 12 min, respectively ($p < 0.05$ and $p < 0.001$) (Figure 1B). Corresponding lag-times were increased 4-, 6-, 10- and 19-fold compared to control. Comparing the potency of Cu-CHL and Mg-CHL on the basis of lag-time increase, Cu-CHL is about 5 times more effective in inhibiting hemin-induced LDL oxidation.

As the LDL anti-oxidative activity of a compound can also depend on its lipophilic/hydrophilic properties, we compared Cu- and Mg-CHL with respect to their octanol/water partition. The results show that Cu-CHL is 2.7-fold ($n = 5$, $p < 0.0001$) more lipophilic than Mg-CHL.

When LDL was pre-incubated with hemin (2.5 μmol/l) for 10 min followed by Cu-CHL and LDL oxidation was started by the addition of reagent H_2O_2, the compound (5 μmol/l) still showed strong antioxidant activity (not shown). Hemin binding to lipoproteins (LDL and HDL) and albumin in serum has been reported [18,19]. Thus we have analyzed the distribution of Cu-CHL and Mg-CHL in comparison to hemin in serum following the protocol of [18]. The visual inspection of the gradients revealed two main colored bands in the lipoprotein region and one at the bottom of the tube (Figure 2A). Figure 2B shows that Cu-CHL and Mg-CHL like hemin can bind to the serum lipoproteins LDL and HDL (and other proteins). Data of three different subjects are depicted. Binding of hemin and the chlorophyllins to isolated LDL and HDL was also estimated by agarose gel electrophoreses as indicated by an increase in REM of the particles (Figure 2C). When LDL was isolated from Cu-CHL or Mg-CHL pre-incubated whole serum, these preparations showed less sensitivity to hemin/H_2O_2-induced LDL oxidation (Figure 3). The transition metal ion (i.e. copper ion) mediated oxidation is a widely used system to study antioxidant compounds [33]. The results in Figure 3 indicate that Cu- and Mg-CHL are also antioxidants in copper-ion induced LDL oxidation. Cu-CHL has been shown to scavenge radicals [39]. In this respect, Mg-CHL in comparison to Cu-CHL showed also radical (DPPH) scavenging activity—although to a lesser extent (see Figure 4). MPO in presence of H_2O_2 and tyrosine can generate tyrosyl radicals initiating lipid oxidation in LDL [14]. When the chlorophyllins were present in the MPO/H_2O_2/tyrosine/LDL oxidizing system both chlorophyllins (5 μmol/l) inhibited tyrosyl radical-mediated LDL oxidation (results not shown). Tyrosyl (phenoxyl) radicals beside their ability to initiate lipid oxidation can form dityrosine via radical–radical reactions (diphenyl formation) [13,38]. When dityrosine formation was monitored in presence of Cu- or Mg-CHL both chlorophyllins (5 μmol/l) showed inhibitory action on diphenyl (dityrosine) formation (Figure 5). Vascular cells (endothelial cells or smooth muscle cells) have the potential to oxidize LDL [40]. Using HUVECs as a model system of cell-mediated LDL oxidation, the results indicate that both chlorophyllins are effective inhibitors of endothelial cell-mediated LDL oxidation (see Figure 6).

In endothelial cells TF activity can be induced by oxidized LDL [41]. When LDL was oxidized by HUVEC and TF activity in cell lysates was measured by a one stage clotting assay, both chlorophyllins were able to counteract TF activity induction by EC-oxidized LDL. Under the conditions employed both Mg- and Cu-CHL showed TF antagonizing potential (Figure 6).

Discussion

LDL oxidation may play a central role in the onset of atherosclerosis [3,42,43]. Thus experimental and

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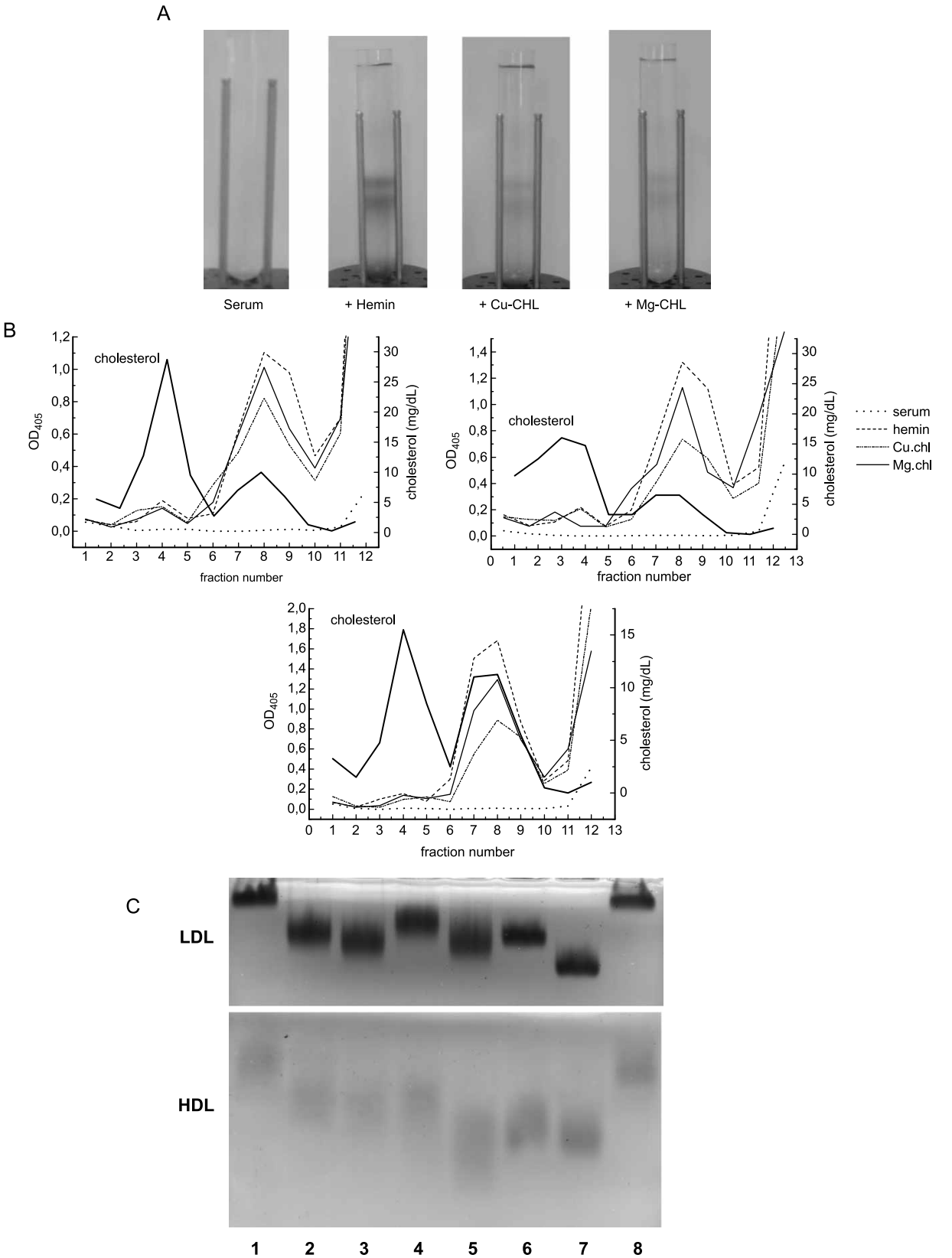

Figure 2. Binding of hemin and CHLs to lipoproteins in serum (A, B) and isolated lipoproteins (C). A: Three different human sera were incubated with hemin or the respective CHL and subjected to KBr gradient ultracentrifugation. A: Visual distribution of the compounds in serum. B: Binding of hemin, Cu-CHL and Mg-CHL to lipoproteins (measured as cholesterol) was estimated as described in "Materials and methods" section. C: LDL or HDL (both 0.5 mg/ml) was incubated in the absence or presence of the respective compound (100 or 50 μmol/l) under the conditions given in "Materials and methods" section. Binding of the compounds was indicated by alteration of the relative electrophoretic mobility. 1: Control; 2: Hemin (50 μmol/l); 3: Hemin (100 μmol/l); 4: Cu-CHL (50 μmol/l); 5: Cu-CHL (100 μmol/l); 6: Mg-CHL (50 μmol/l); 7: Mg-CHL (100 μmol/l); 8: Control.

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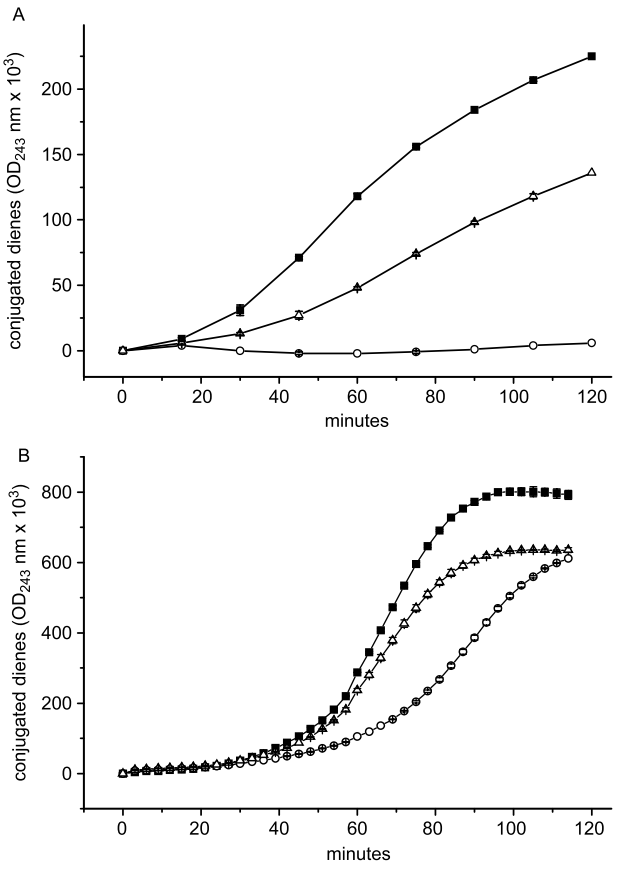

Figure 3. Lipid oxidation in LDL isolated from serum pre-incubated with Cu-CHL or Mg-CHL. A: Hemin/H_2O_2 induced LDL oxidation. B: Copper ion-induced LDL oxidation. Whole serum was incubated with or without CHLs (125 μmol/l) for 15 min and LDL was isolated by ultracentrifugation as given in "Materials and methods" section. LDL (0.2 mg/ml) was oxidized in presence of 2.5 μmol/l hemin/40 μmol/l H_2O_2 or 5 μmol/l Cu^{++} at 37°C. Control:■. Mg-CHL:△. Cu-CHL:○.

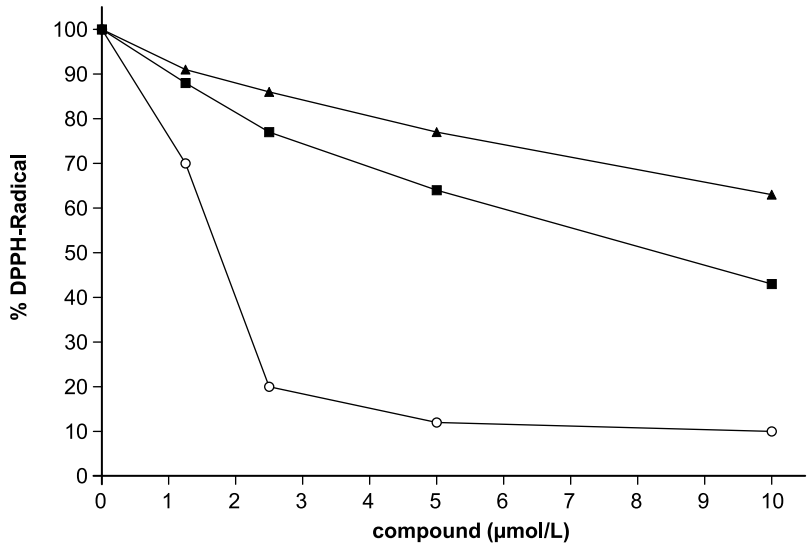

Figure 4. DPPH radical scavenging ability of Cu-CHL or Mg-CHL. Cu-CHL or Mg-CHL were added to a solution of DPPH radical (50 μmol/l). After 10 min the decrease in absorption at 517 nm was taken as indicator of radical scavenging ability. Pyrogallol was used as a positive control. Pyrogallol:○. Cu-CHL:■. Mg-CHL:▲.

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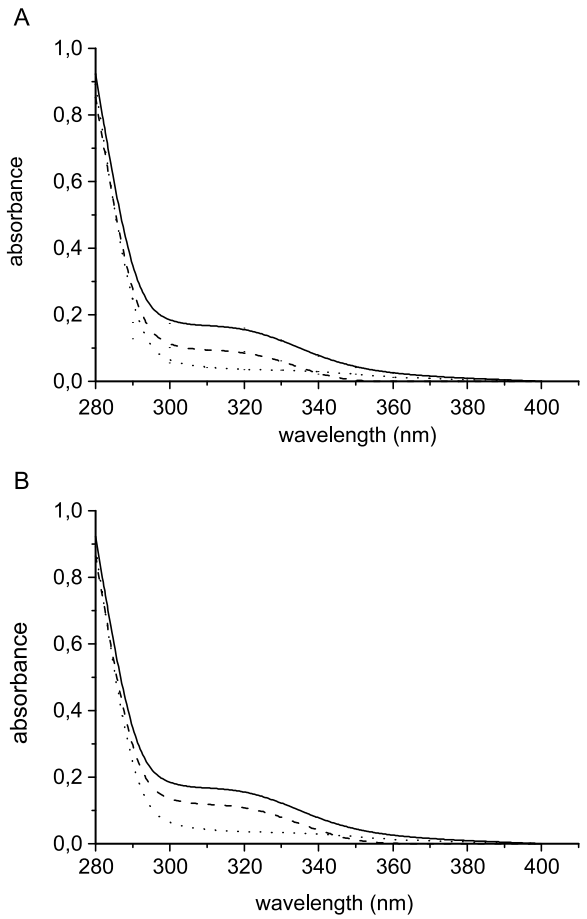

Figure 5. Influence of Cu-CHL (A) or Mg-CHL (B) on dityrosine formation. Phenoxyl radical-mediated dityrosine (diphenyl) formation in absence or presence of CHLs (5 μmol/l) was followed spectroscopically as given in "Materials and methods" section. Tyrosine/MPO/H_2O_2: ____; tyrosine/H_2O_2: ……; Tyrosine/MPO/H_2O_2 + CHL: – – – –.

clinical investigations focused on the action and protective effect of natural and synthetic antioxidative compounds. Although, a "Janus-faced" action i.e. anti- and pro-oxidative action of these compounds can not be ruled out as recently stressed by Halliwell [44]. Cu-CHLs have been especially identified as potent anticancerogenic substances [45–48] and in addition as compounds with antioxidant activity in liposomal and biological membrane systems (mitochondria) exposed to various radical generating reactions [27–29]. Cu-CHL is the copper-sodium salt and water-soluble analog of chlorophyllin. Cu-CHL is widely used as food coloring agent, health food additive, supporter of wound healing and control of fecal and urinary odor in colostomy patients [49]. Therapeutic levels of about 3 μmol Cu-CHL/l plasma without any toxic effects have been reported in the study of Egner et al. [46]. The antimutagenic [50], antigenotoxic [51] and anticarcinogenic [52] activity of the compound may be attributed to the ability of CHL to form tight complexes with the respective mutagenic, carcinogenic or genotoxic molecules. Due to their hydrophobic and negatively charged porphyrin ring system these compounds may bind to serum lipoproteins like LDL and HDL, as has been demonstrated for hemin, which has a similar porphyrin ring system [18,19]. Thus one may speculate that chlorophyllins may have the potential to act as antioxidants in reactions initiating LDL oxidation especially by hemin. In accordance to these observations the present results show that copper- and Mg-CHL are potent antioxidative compounds in LDL oxidation reactions induced by hemin. In addition, the CHLs inhibited the transition metal ion, tyrosyl radical and endothelial cell mediated LDL oxidizing systems. In the present study as low as 1 μmol/l chlorophyllin showed inhibitory action on LDL atherogenic modification, a concentration which is well in the range achievable in humans after oral uptake [50]. Mg-CHL in all systems showed slightly less antioxidant activity which might be due to its lower lipophilicity (measured as Octanol/water partition) compared to Cu-CHL. In the MPO catalyzed LDL oxidation reaction which depends on H_2O_2 and tyrosine, both chlorophyllins were found to inhibit lipid oxidation. This may be due to scavenging (repairing) the tyrosyl radical or inhibiting MPO as indicated by reduced dityrosine formation. The copper ion mediated LDL oxidation may be inhibited by complexing Cu^{2+} by the porphyrin ring system via the carboxylic acid residues. It should be noted that Cu-CHL and Mg-CHL could also suppress lipoprotein lipid oxidation during the propagation phase. Hence, the chlorophyllins may have chain-breaking ability in addition to their inhibitory action on the initiation reaction of lipid oxidation. On the other hand, the central copper in Cu-CHL may be redox-active. Thus the compound may act by an electron-transfer mechanism by quenching the activated heme—presumably an oxo-ferryl heme porphyrin radical cation—or protect LDL by scavenging lipid radicals.

TF plays a role in the late phase of atherosclerosis (i.e. in thrombus formation of ruptured atherosclerotic plaques) and oxidized LDL has been shown to induce TF activity [41]. Using a functional assay, we demonstrated that the chlorophyllins—due to their antioxidative effects—could counteract TF activity induction by oxidized LDL.

It should be kept in mind that not all antioxidants that inhibit LDL oxidation *in vitro* are necessarily effective also *in vivo* [53]. In this respect Upston et al. have shown that LDL oxidation in the sub-endothelial space apparently takes place in the presence of vitamin E which is an effective antioxidant *in vitro*.[54,55]

In summary, the results show that the chlorophyllins are potent antioxidants in LDL oxidizing systems like hemin, transition metal ions, endothelial cells, and

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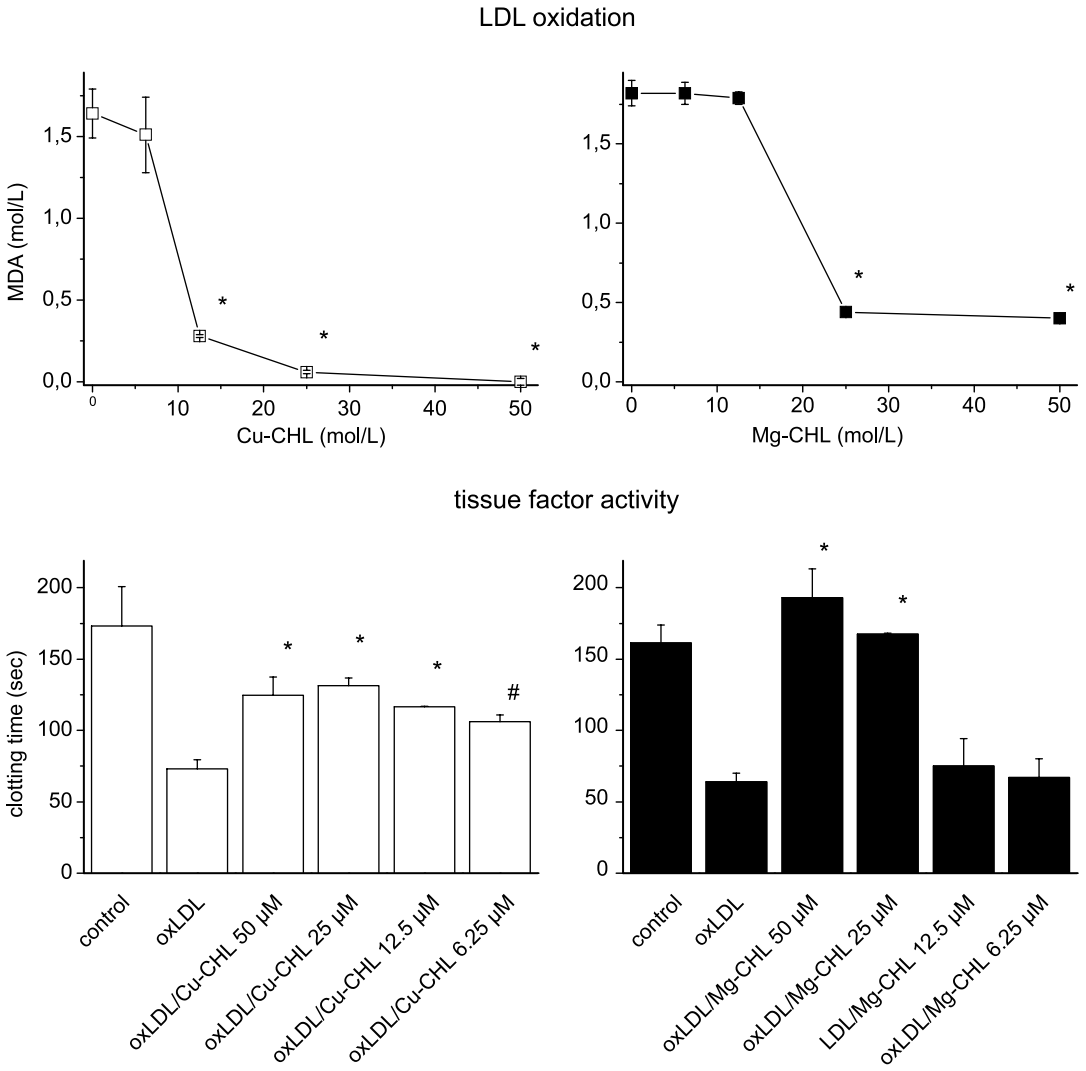

Figure 6. Influence of CHLs on endothelial cell mediated LDL oxidation (upper panel). LDL (0.1 mg/ml) was oxidized for 18 h by HUVECs in the absence or presence of Cu-CHL (left panel, □) or Mg-CHL (right panel, ■) (both 6.25–50 µmol/l). LDL oxidation was monitored as TBARS formation and expressed as malondialdehyde equivalents. Values represent mean ± SD of 3 experiments ($^{*}p < 0.001$ vs. control). Effect of CHLs on endothelial tissue factor activity (lower panel). HUVECs were treated as described above to oxidize LDL and tissue factor activity was subsequently measured by a one-stage clotting assay as described in "Materials and methods" section. Values represent mean ± SD of 3 experiments ($^{*}p < 0.001$ vs. oxLDL, $^{\#}p < 0.01$ vs. oxLDL).

MPO catalyzed reactions. Our results obtained with isolated human LDL support the findings of [56] who found less atherosclerotic alterations in vessel walls of rats receiving an atherosclerotic diet in presence of Cu-CHL.

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