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Slow oxidation of high density lipoproteins as studied by EPR spectroscopy

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Abstract
There is relatively little information on the role of high density lipoprotein (HDL) oxidation in atherogenesis although there are indications that oxidation might affect atheroprotective activities of HDL. Recently we reported the study on LDL oxidation initiated and sustained by traces of the transition metal ions under conditions, which favor slow oxidation. Here we report the results of the analogous study on the oxidation of the two HDL subclasses. The oxidation process was monitored by measuring the time dependence of oxygen consumption and concentration of the spin-trapped free radicals using EPR spectroscopy. In both HDL_2 and HDL_3 subclasses, the dependence of the oxidation process on the copper/lipoprotein molar ratio is different from that in LDL dispersions. Comparison of the kinetic profiles of HDL_2 and HDL_3 oxidation revealed that under all studied experimental conditions HDL_2 was more susceptible to copper-induced oxidation than HDL_3.

Keywords: *High density lipoprotein, slow oxidation, copper ions, EPR spectroscopy*

Introduction

Numerous epidemiological and clinical studies have demonstrated inverse and independent relationship between high density lipoprotein cholesterol (HDL-C) level and the risk for cardiovascular disease [1]. However, it is not known whether this relationship is causal [2,3]. The association between low HDL-C level and an increased risk for coronary artery disease has been explained by antiatherogenic activities of HDL, including its role in reverse cholesterol transport (RCT), its antioxidant and anti-inflammatory properties [4,5]. High density lipoprotein is a class of plasma lipoproteins characterized by high density and small size. HDL particles are heterogeneous in physicochemical properties, intravascular metabolism and biological activity [3,6]. It has been suggested that the concentration of various HDL subclasses, not the total HDL-C concentration, and the kinetics of HDL metabolism are important determinants of the HDL anti-atherogenic activities and the risk of atherosclerosis, respectively [3,4]. It is now quite generally accepted that oxidatively modified low density lipoprotein (LDL) is implicated in the initiation and progression of atherosclerosis, a chronic inflammatory disease [7]. The role of HDL in inflammation is more complex. In its basal state, HDL is anti-inflammatory, but during acute inflammation HDL become pro-oxidant [8]. HDL can also undergo oxidative modification *in vivo* and HDL lipids are equally or even more susceptible to oxidation than those of LDL [9–12]. Compared to LDL, relatively little is known about the role of HDL oxidation in atherogenesis. There is also little

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information on relative susceptibility to oxidation of different HDL subclassess. Oxidative modification of HDL might have important consequences concerning the efficiency of HDL in promoting cholesterol efflux from the peripheral cells (RTC) and in inhibiting LDL oxidation [11,13]. Therefore, studying the mechanisms of the HDL oxidation might have potential pathophysiological significance.

Like LDL, oxidation of HDL has been studied *in vitro* using variety of initiators including H_2O_2, myeloperoxidase-derived oxidants, copper, lipooxygenase and peroxyl radicals [11,14]. Copper ions are commonly used since metal ions may contribute to lipoprotein oxidation at least in the late stages of atherosclerotic lesion development [11,15]. In our earlier studies, we demonstrated that copper could also initiate oxidation of LDL in a closed system with a defined and limited amount of oxygen and with very small concentration of copper ions (the Cu^{2+} ion to LDL molar ratio was within the range of $0.01 - 0.2$) [16,17]. The main features of the temporal behavior of several oxidation markers (oxygen consumption, free radical generation, lipid hydroperoxide formation and α-tocopherol concentration) were explained in terms of the tocopherol-mediated peroxidation (TMP), initiated by traces of transition metal ions associated with LDL particles [18,19]. The developed probabilistic kinetic model could simulate slow and mild LDL oxidation under unfavorable oxidizing conditions that could be physiologically relevant. There is also evidence that TMP might be relevant for HDL oxidation [20–22]. That promted us to investigate the application of the probabilistic kinetic model based on TMP to study copper-initiated oxidation of the HDL_2 and HDL_3 subclasses.

Dynamics of copper-induced HDL oxidation carried out under unfavourable oxidizing conditions was followed by measuring oxygen consumption and concentration of trapped free radicals in a closed system. The study on LDL oxidation has demonstrated that oxygen consumption is the best indicator of the oxidation progress in lipoprotein dispersions [17].

Materials and methods

Chemicals

2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPO), diphenyl-picryl-hydrazyl (DPPH), a 3-carbamoyl-2,2,5,5-tetramethyl-3-pyroline-1-yloxy (CTPO) spin probe and a *N*-*t*-butyl-α-phenylnitrone (PBN) spin trap were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Potassium bromide (KBr) p.a. and copper (II) chloride dihydrate ($CuCl_2 \cdot 2H_2O$) p.a. were obtained from Merk (Darmstadt, Germany). All other chemicals for buffer preparation (NaCl, KCl, Na_2HPO_4, KH_2PO_4) and

lipoprotein isolation were of the highest purity grade available and were purchased from Sigma-Aldrich. Aqueous solutions for all experiments were prepared using ultrapure water obtained from a SG ultra clear water purification system (SG Wasseraufbereitung und Regenerierstation GmbH; Barsbüttel, Germany).

HDL isolation and oxidation

HDL_2 ($d = 1.063 - 1.125$ g/ml) and HDL_3 ($d = 1.125 - 1.210$ g/ml) subclasses were isolated from plasma of healthy female blood donors in presence of ethylenediaminetetraacetic acid (EDTA) (1 mg/ml) by sequential ultracentrifugation using Beckman Optima™ LE-80K ultracentrifuge equipped with a 70.1 Ti rotor (Beckman Instruments, Palo Alto, CA, USA) [23]. Before copper oxidation, isolated HDL_2 and HDL_3 subfractions were dialyzed at 4°C for 24 h against 0.15 M phosphate-buffered saline (PBS), pH 7.4, to remove salts and EDTA. Subsequently, HDL preparations were concentrated using Minicon®-B15 concentrators (Millipore Corporation, Bedford, MA, USA) and then adjusted to the desired concentration with buffer (PBS) or buffered solutions of the spin probe (CTPO) or spin trap (PBN). The lipoprotein concentrations were assessed by determination of total dry mass of the preparations, after removal of all salts by dialysis, assuming the relative molecular mass of 360,000 for HDL_2 and 175,000 for HDL_3. The purity of the HDL preparations was checked by lipoprotein electrophoresis kit (Lipidophor all in 12; Immuno AG, Vienna, Austria).

Prior to oxidation at 37°C, freshly prepared $CuCl_2$ solution ($2 - 4$ μmol/l) was added to the HDL_2 and HDL_3 dispersions ($28 - 114$ μmol/l). Some HDL samples were oxidized without adding any $CuCl_2$. Oxidation was carried out in tightly closed quartz capillaries (Drummond Scientific Company; Broomal, PA, USA) to prevent oxygen diffusion from the atmosphere into the samples.

The Cu^{2+} ion to HDL molar ratio was within the range of $0.02 - 0.14$.

Free radical detection

Oxidation-induced free radicals in the HDL_2 and HDL_3 samples were recorded with the electron paramagnetic resonance (EPR) spectroscopy using PBN dissolved in PBS as a spin trap. PBN was added to the HDL samples prior to incubation at final concentration of 50 mM. The EPR spectra were recorded at 37°C on a Varian E-109 spectrometer, equipped with a variable temperature control unit. The quantitative estimate of the free radical content in the HDL samples was done by comparing the HDL spectra with the spectra of known concentration of the stable radical (TEMPO) after double integration.

Measurement of oxygen consumption

Oxygen concentration in the HDL_2 and HDL_3 samples was measured by EPR oxymetry using a CTPO spin probe [24]. EPR spectrum of CTPO added to the HDL dispersions immediately before incubation at 37°C is sensitive to the concentration of oxygen in an aqueous phase of the sample [16].

Results and discussion

To investigate the effect of copper content on the dynamics of HDL oxidation, samples of HDL_2 and HDL_3 subclasses with varying copper concentration were prepared. In these experiments, the highest molar ratio of copper to HDL was 0.14, the value much lower than in most other studies of copper-induced HDL oxidation [21,22,25]. It has been demonstrated that transition metal ions are ubiquitous in the contamination of *in vitro* systems [26]. It means that traces of the transition metal ions were present even in the HDL samples with no copper ions purposely added.

The change of oxygen concentration in the aqueous phase of the HDL samples with the incubation time is shown in Figure 1. The results are typical of six independent experiments performed with the samples of different donors. The measurements were made for the HDL samples ($\approx 56\,\mu mol/l$) with no copper ions added (triangles), with $2\,\mu mol/l$ (squares) and $4\,\mu mol/l$ of copper ions added (circles). The data for HDL_2 are shown in panel A and those for HDL_3 in panel B. The curves presented in the figure were calculated by the polynomial fitting procedure. The experimental data demonstrate that in both HDL subclasses the oxidation measured by the rate of oxygen consumption is accelerated with the increase of the copper content. Since oxygen is consumed in the lipid peroxidation chain reactions, these results confirm the involvement of the copper in both initiation and propagation of the HDL oxidation. Figure 1 also demonstrates that the oxidation process is faster in HDL_2 than in HDL_3 samples. If samples of equal molar or mass concentration are compared, the rate of HDL_2 oxidation is higher than the rate of HDL_3 oxidation within the studied range of copper concentration. That difference is somewhat less pronounced in the samples without purposely added copper, presumably due to much lower intrinsic concentration of the metal ions.

Similar differences between the two subclasses of HDL were observed by measuring concentration of induced free radicals. The increase in concentration of the trapped free radicals with time of incubation is presented in Figure 2. In both panels the curves for the samples with no added (triangles) and those with $4\,\mu mol/l$ added copper (circles) are presented. Panel A

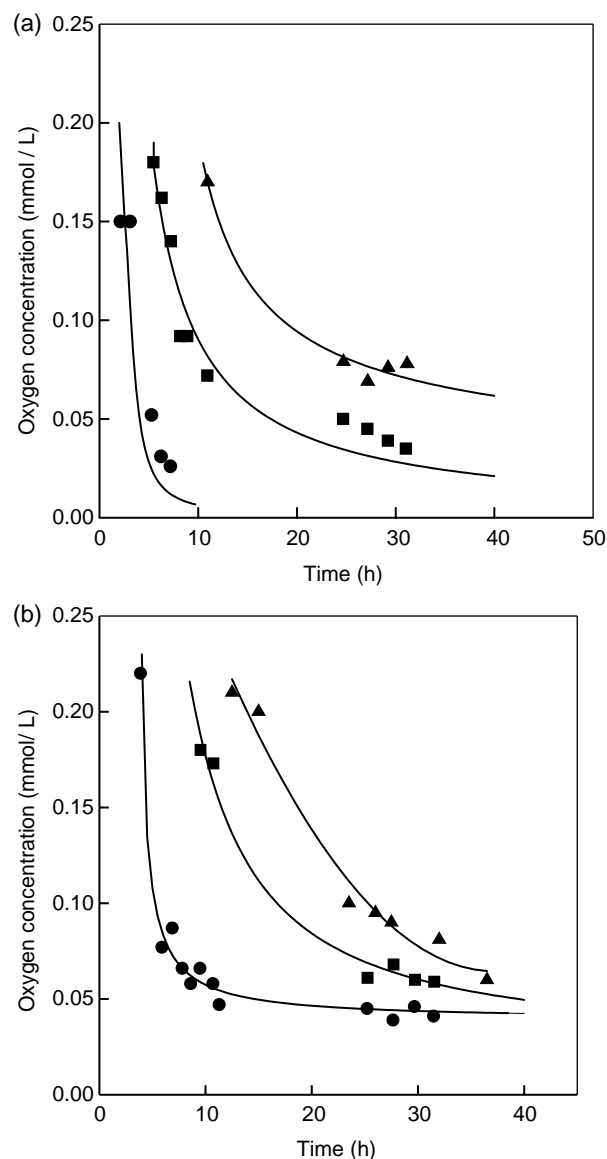Figure 1. Panel A: Time dependence of oxygen consumption in HDL_2 ($56\,\mu mol/l$) without added Cu^{2+} ions (▲) and with added $4\,\mu mol/l$ (●) and $2\,\mu mol/l$ (■) of copper. Panel B: Time dependence of oxygen consumption in HDL_3 ($57\,\mu mol/l$) without added Cu^{2+} ions (▲) and with added $2\,\mu mol/l$ (■) and $4\,\mu mol/l$ (●) of copper ions, respectively. Data shown represent a typical experiment of four different HDL preparations.

refers to HDL_2, and panel B to HDL_3. The presence of PBN-spin adducts in EPR spectra was detected only after a significant amount of oxygen was consumed, as could be observed by comparing Figure 1 and 2. That implicates an efficient radical–radical recombination which results in the formation of stabilized products in non-radical form. Similar behaviour was observed for LDL oxidation [16]. The trapped free radicals could be located in the lipid or protein domain of the HDL particles. In our earlier study of LDL oxidation [16] about 90% of spin adducts were found to be protein-bound. Since the protein/lipid ratio in HDL is higher than in LDL and
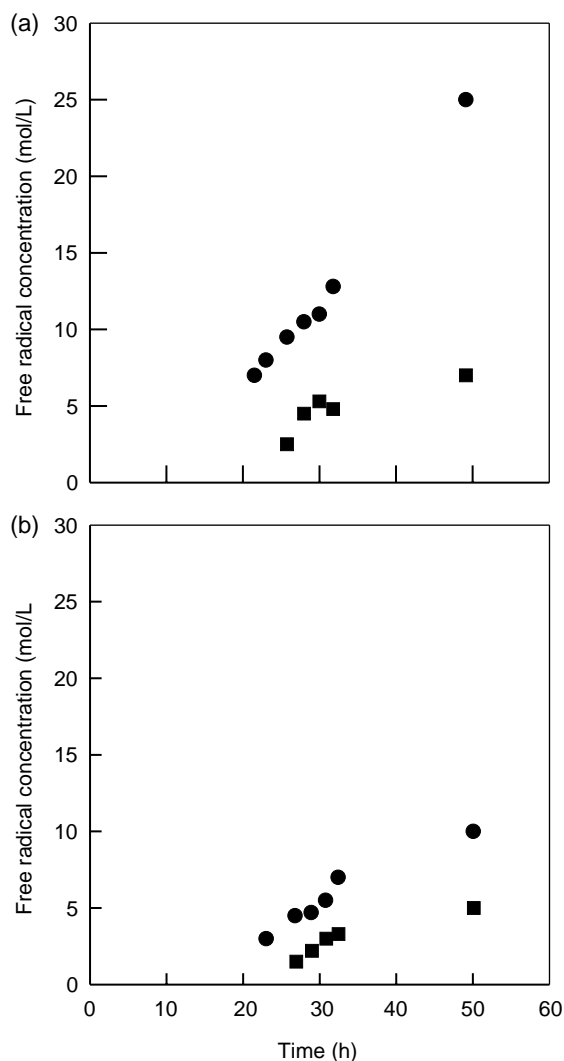
Figure 2. Accumulation of PBN-trapped free radicals with time of incubation. Panel A: HDL$_2$ (56 µmol/l) without the added Cu^{2+} ions (■) and with Cu^{2+} ions added (4 µmol/l (●)). Panel B: HDL$_3$ (57 µmol/l) without added Cu^{2+} ions (■) and with Cu^{2+} ions added (4 µmol/l (●)).

because HDL-associated apolipoproteins AI and II are oxidized by lipid hydroperoxides during mild oxidation of HDL [14], it is probable that the percentage of the protein-bound trapped radicals in HDL is even higher. No attempt was made to prove that.

The dependence of the rate of oxygen consumption on HDL concentration is shown in Figure 3. Panel A shows the data for HDL$_2$ (56 µmol/l—circles and 28 µmol/l—squares) and panel B the data for HDL$_3$, (114 µmol/l—circles and 29 µmol/l—squares). Contrary to our expectations based on the model of LDL oxidation [19], oxygen was consumed faster in the samples of lower HDL concentration for both HDL subclasses. Such a behaviour was observed in four independent experiments performed with the HDL samples of different donors. However, the free radical accumulation did not behave in the same way, as
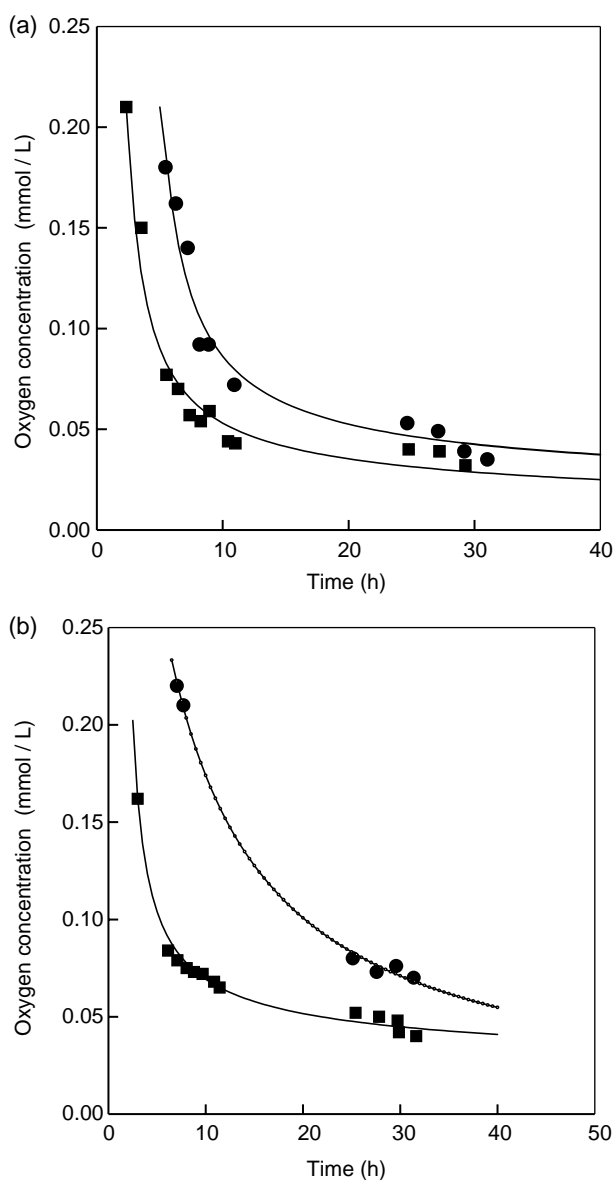

Figure 3. Representative data on the effect of copper (2 µmol/L) on the rate of oxygen consumption in the HDL$_2$ samples of two different concentration: 28 µmol/L (■) and 56 µmol/L (●) (Panel A) and on the rate of oxygen consumption in the HDL$_3$ samples of two different concentration: 29 µmol/L (■) and 114 µmol/L (●) (Panel B).

shown in Figure 4. For both HDL subclasses the rate of free radical accumulation was faster in the samples of higher HDL concentration. In the figure, panel A shows the data for HDL$_2$ (56 µmol/l—circles and 28 µmol/l—squares) and panel B for HDL$_3$ (114 µmol/l—circles and 57 µmol/l—squares). Apparently different behaviour of the two oxidation markers during slow and mild oxidation of the HDL could be the result of interparticle interaction, i.e. due to transfer of the oxidation products from one particle to another. It is quite probable that most of the free radicals carrying lipid peroxidation chain reactions are stabilized in the protein domain of the
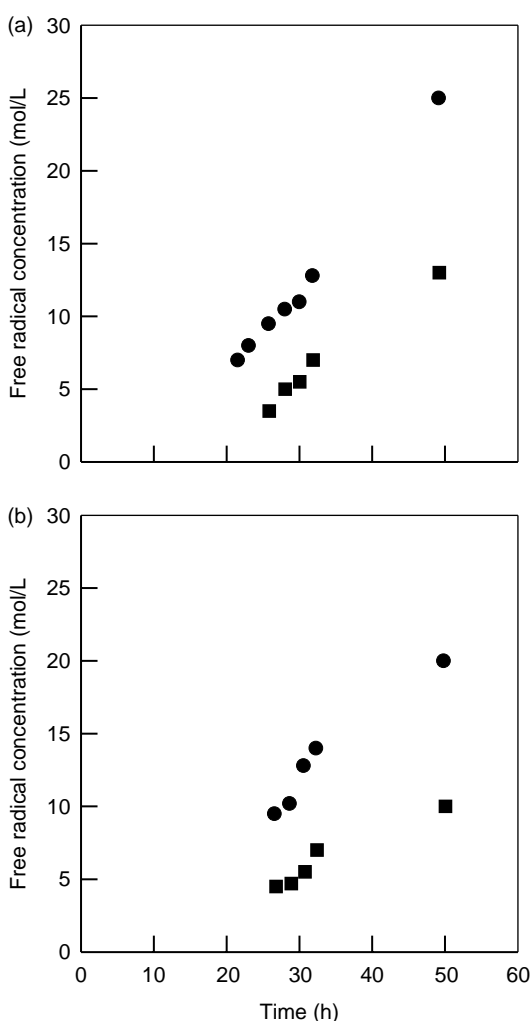
Figure 4. The time dependence of PBN-trapped free radicals in the HDL_2 samples of concentrations: 28 μmol/l (■) and 56 μmol/l (●) (Panel A) and HDL_3 samples of concentrations: 57 μmol/l (■) and 114 μmol/l (●) (Panel B). The copper content in the samples was 4 μmol/l.

HDL particle or end up as non-radical products in the recombination reactions. If so, the total number of PBN-trapped free radicals is expected to be higher in the samples of higher HDL concentration. On the other hand, in the dispersions of higher HDL concentration the formed lipid hydroperoxyl radicals have higher probability to directly react with the surface proteins on the neighbouring HDL particles, thus avoiding reaction with oxygen. In accordance with this line of reasoning is the observation that in mildly oxidized HDL the apolipoproteins dissociate more easily from the particles than in native HDL [14].

    To conclude, it is obvious that the copper-induced oxidation of HDL is a rather complex process and that our theoretical model of LDL oxidation [19] is not appropriate for the description of the kinetics of the HDL oxidation. The probabilistic kinetic model is based on the concept of LDL particles as

distinctive compartments with only weak inter-particle interaction between the oxidation bursts. To model the oxidation of the HDL dispersions, the intra-particle and inter-particle events should be taken into account at the same level of calculation.

Acknowledgements

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