

## 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<sub>2</sub> and HDL<sub>3</sub> 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<sub>2</sub> and HDL<sub>3</sub> oxidation revealed that under all studied experimental conditions HDL<sub>2</sub> was more susceptible to copper-induced oxidation than HDL<sub>3</sub>.

**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 subclasses. 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 peroxy 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  $\alpha$ -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 prompted us to investigate the application of the probabilistic kinetic model based on TMP to study copper-initiated oxidation of the HDL<sub>2</sub> and HDL<sub>3</sub> 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-pyrroline-1-yloxy (CTPO) spin probe and a *N*-*t*-buthyl- $\alpha$ -phenylnitron (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<sub>2</sub> ( $d = 1.063 - 1.125$  g/ml) and HDL<sub>3</sub> ( $d = 1.125 - 1.210$  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<sub>2</sub> and HDL<sub>3</sub> 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<sub>2</sub> and 175,000 for HDL<sub>3</sub>. 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  $\mu$ mol/l) was added to the HDL<sub>2</sub> and HDL<sub>3</sub> dispersions (28 – 114  $\mu$ mol/l). Some HDL samples were oxidized without adding any  $CuCl_2$ . Oxidation was carried out in tightly closed quartz capillaries (Drummond Scientific Company; Broomall, 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<sub>2</sub> and HDL<sub>3</sub> 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<sub>2</sub> and HDL<sub>3</sub> 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<sub>2</sub> and HDL<sub>3</sub> 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 ( $\approx 56 \mu\text{mol/l}$ ) with no copper ions added (triangles), with  $2 \mu\text{mol/l}$  (squares) and  $4 \mu\text{mol/l}$  of copper ions added (circles). The data for HDL<sub>2</sub> are shown in panel A and those for HDL<sub>3</sub> 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<sub>2</sub> than in HDL<sub>3</sub> samples. If samples of equal molar or mass concentration are compared, the rate of HDL<sub>2</sub> oxidation is higher than the rate of HDL<sub>3</sub> 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  $4 \mu\text{mol/l}$  added copper (circles) are presented. Panel A

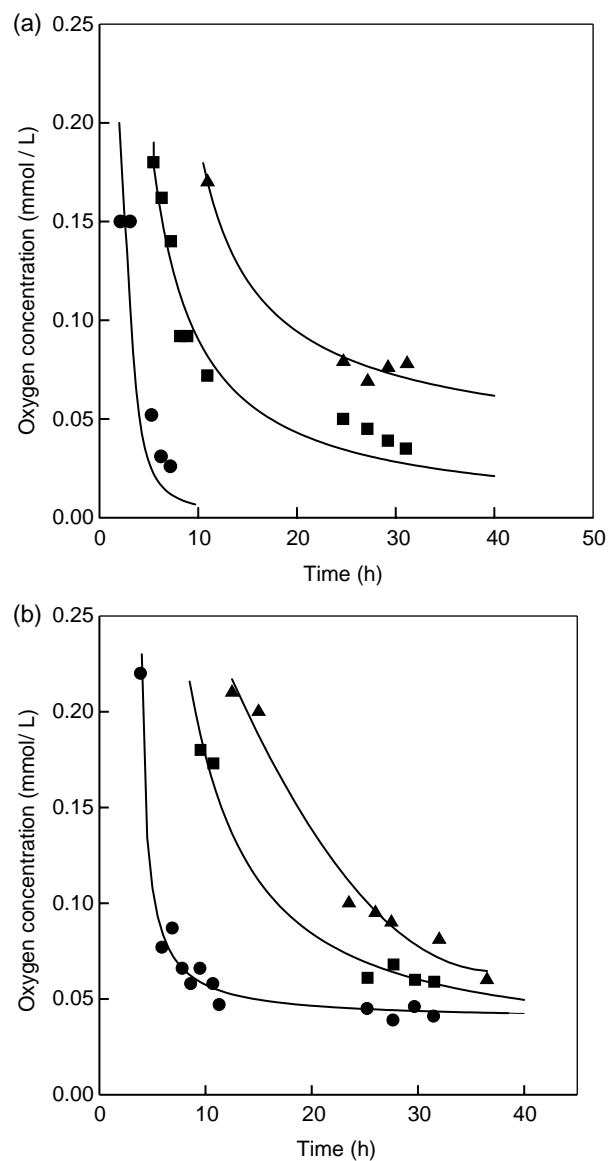


Figure 1. Panel A: Time dependence of oxygen consumption in HDL<sub>2</sub> ( $56 \mu\text{mol/l}$ ) without added  $\text{Cu}^{2+}$  ions ( $\blacktriangle$ ) and with added  $4 \mu\text{mol/l}$  ( $\bullet$ ) and  $2 \mu\text{mol/l}$  ( $\blacksquare$ ) of copper. Panel B: Time dependence of oxygen consumption in HDL<sub>3</sub> ( $57 \mu\text{mol/l}$ ) without added  $\text{Cu}^{2+}$  ions ( $\blacktriangle$ ) and with added  $2 \mu\text{mol/l}$  ( $\blacksquare$ ) and  $4 \mu\text{mol/l}$  ( $\bullet$ ) of copper ions, respectively. Data shown represent a typical experiment of four different HDL preparations.

refers to HDL<sub>2</sub>, and panel B to HDL<sub>3</sub>. 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

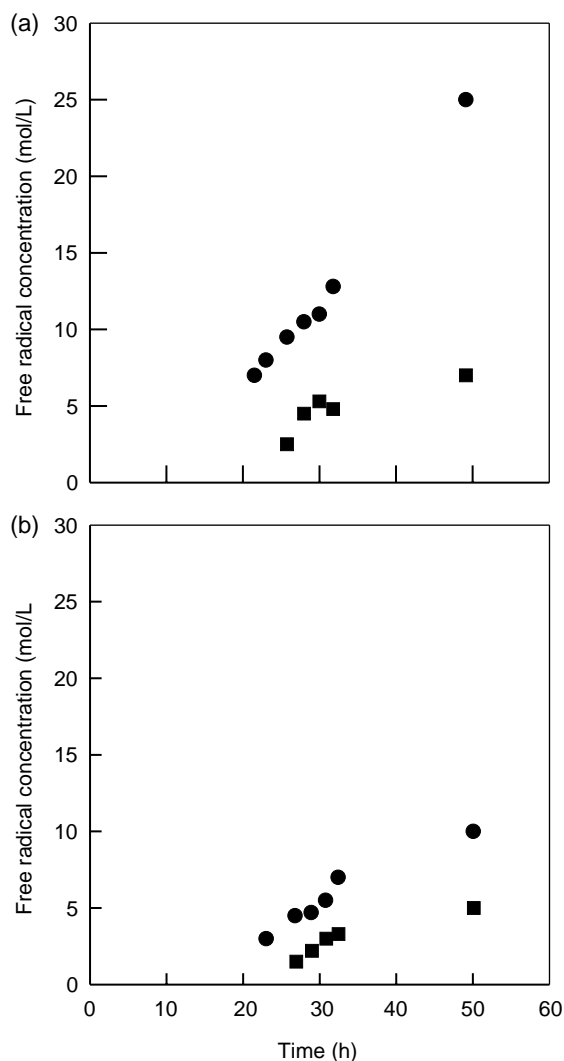


Figure 2. Accumulation of PBN-trapped free radicals with time of incubation. Panel A: HDL<sub>2</sub> (56  $\mu\text{mol/l}$ ) without the added  $\text{Cu}^{2+}$  ions (■) and with  $\text{Cu}^{2+}$  ions added (4  $\mu\text{mol/l}$ ) (●). Panel B: HDL<sub>3</sub> (57  $\mu\text{mol/l}$ ) without added  $\text{Cu}^{2+}$  ions (■) and with  $\text{Cu}^{2+}$  ions added (4  $\mu\text{mol/l}$ ) (●).

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<sub>2</sub> (56  $\mu\text{mol/l}$ —circles and 28  $\mu\text{mol/l}$ —squares) and panel B the data for HDL<sub>3</sub>, (114  $\mu\text{mol/l}$ —circles and 29  $\mu\text{mol/l}$ —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

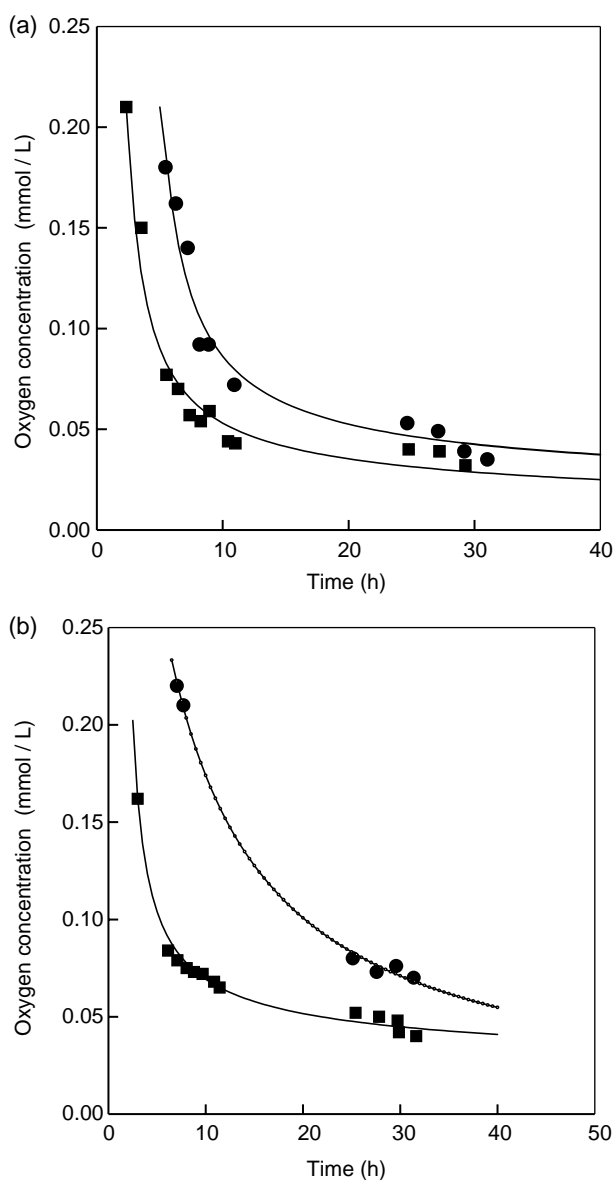


Figure 3. Representative data on the effect of copper (2  $\mu\text{mol/L}$ ) on the rate of oxygen consumption in the HDL<sub>2</sub> samples of two different concentration: 28  $\mu\text{mol/L}$  (■) and 56  $\mu\text{mol/L}$  (●) (Panel A) and on the rate of oxygen consumption in the HDL<sub>3</sub> samples of two different concentration: 29  $\mu\text{mol/L}$  (■) and 114  $\mu\text{mol/L}$  (●) (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<sub>2</sub> (56  $\mu\text{mol/l}$ —circles and 28  $\mu\text{mol/l}$ —squares) and panel B for HDL<sub>3</sub> (114  $\mu\text{mol/l}$ —circles and 57  $\mu\text{mol/l}$ —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

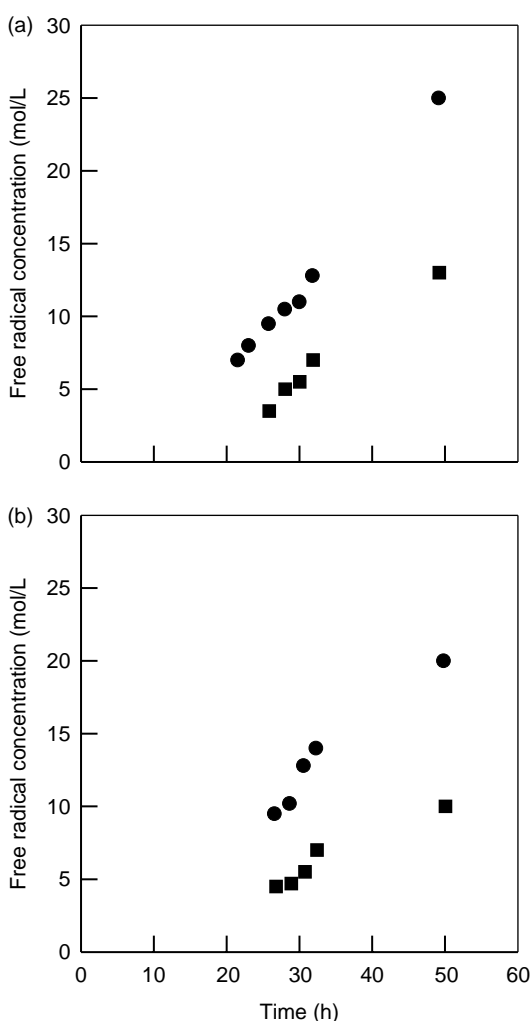


Figure 4. The time dependence of PBN-trapped free radicals in the HDL<sub>2</sub> samples of concentrations: 28  $\mu\text{mol/l}$  (■) and 56  $\mu\text{mol/l}$  (●) (Panel A) and HDL<sub>3</sub> samples of concentrations: 57  $\mu\text{mol/l}$  (■) and 114  $\mu\text{mol/l}$  (●) (Panel B). The copper content in the samples was 4  $\mu\text{mol/l}$ .

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.

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### References

- [1] Gordon DJ, Rifkind BM. High-density lipoproteins: The clinical implications of recent studies. *N Engl J Med* 1989;321:1311–1316.
- [2] Navab M, Ananthramaiah GM, Reddy ST, Van Lenten BJ, Ansell BJ, Fonarow GC, Vahabzadeh K, Hama S, Hough G, Kamranpour N, Berliner JA, Lusis AJ, Fogelman AM. The oxidation hypothesis of atherogenesis: The role of oxidized phospholipids and HDL. *J Lipid Res* 2004;45:993–1007.
- [3] Assmann G, Gotto AM. HDL cholesterol and protective factors in atherosclerosis. *Circulation (suppl III)* 2004;III-8–III-14.
- [4] Von Eckardstein A, Nofer J-R, Assmann G. High density lipoproteins and atherosclerosis. Role of cholesterol efflux and reverse cholesterol transport. *Arterioscler Thromb Vasc Biol* 2001;21:13–27.
- [5] Nofer J-R, Kehrel B, Fobker M, Levkau B, Assmann G, von Eckardstein A. HDL and arteriosclerosis: Beyond reverse cholesterol transport. *Atherosclerosis* 2002;161:1–16.
- [6] Kontush A, Chantepie S, Chapman MJ. Small, dense HDL particles exert potent protection of atherogenic LDL against oxidative stress. *Arterioscler Thromb Vasc Biol* 2003;23:1881–1888.
- [7] Witztum JL, Steinberg D. Role of oxidized low density lipoprotein in atherogenesis. *J Clin Invest* 1991;88:1785–1792.
- [8] Van Lenten BJ, Navab M, Shih D, Fogelman AM, Lusis AJ. The role of high-density lipoproteins in oxidation and inflammation. *Trends Cardiovasc Med* 2001;11:155–161.
- [9] Bowry W, Stanley KK, Stocker R. High density lipoprotein is the major carrier of lipid hydroperoxides in fasted human plasma. *Proc Natl Acad Sci USA* 1992;89:10316–10320.
- [10] Nin X, Zammit V, Upston JM, Dean RT, Stocker R. Co-existence of oxidized lipids and  $\alpha$ -tocopherol in all lipoprotein fractions isolated from advanced human atherosclerotic plaques. *Arterioscler Thromb Vasc Biol* 1999;19:1708–1718.
- [11] Francis GA. High density lipoprotein oxidation: *In vitro* susceptibility and potential *in vivo* consequences. *Biochim Biophys Acta* 2000;1483:217–235.
- [12] Pennathur S, Bergt C, Shao B, Byun J, Kassim SY, Singh P, Green PS, McDonald TO, Brunzell J, Chait A, Oram JF, O'Brien K, Geary RL, Heinecke JW. Human atherosclerotic intima and blood of patients with established coronary artery disease contain high density lipoprotein damaged by reactive nitrogen species. *J Biol Chem* 2004;279:42977–42983.
- [13] Bergt C, Pennathur S, Fu S, Byun J, O'Brien K, McDonald TO, Singh P, Ananthramaiah GM, Chait A, Brunzell J, Geary RL, Oram JF, Heinecke JW. The myeloperoxidase product hypochlorous acid oxidizes HDL in the human artery wall and impairs ABCA1-dependent cholesterol transport. *Proc Natl Acad Sci USA* 2004;101:13032–13037.
- [14] Pankhurst G, Wang XL, Wilcken DE, Baerenthaler G, Panzenböck U, Raftery M, Stocker R. Characterization of specifically oxidized apolipoproteins in mildly oxidized high density lipoprotein. *J Lipid Res* 2003;44:349–355.

- [15] Gaut JP, Heinecke JW. Mechanisms for oxidizing low-density lipoprotein. Insights from patterns of oxidation products in the artery wall and from mouse models of atherosclerosis. *Trends Cardiovasc Med* 2001;11:103–112.
- [16] Herak JN, Krilov D, Stojanović N, Marincel J. Very slow autooxidation of low-density lipoprotein spares  $\alpha$ -tocopherol. *Chem Phys Lipids* 1998;94:63–70.
- [17] Krilov D, Stojanović N, Herak JN. Probabilistic kinetic model of slow oxidation of low-density lipoprotein. II. Experiments. *Chem Phys Lipids* 2004;129:75–84.
- [18] Bowry VW, Stocker R. Tocopherol-mediated peroxidation. The prooxidant effect of vitamin E on the radical-initiated oxidation of human low-density lipoprotein. *J Am Chem Soc* 1993;115:6029–6044.
- [19] Herak JN, Stojanović N, Krilov D. Probabilistic kinetic model of slow oxidation of low-density lipoprotein. I. Theory. *Chem Phys Lipids* 2004;129:63–74.
- [20] Garner B, Witting PK, Waldeck AR, Christison JK, Raftery M, Stocker R. Oxidation of high density lipoproteins I. Formation of methionine sulfoxide in apolipoproteins AI and AII is an early event that accompanies lipid peroxidation and can be enhanced by  $\alpha$ -tocopherol. *J Biol Chem* 1998;273:6080–6087.
- [21] Raveh O, Pinchuk I, Schnitzer E, Fainaru M, Schaffer Z, Lichtenberg D. Kinetic analysis of copper-induced peroxidation of HDL, autoaccelerated and tocopherol-mediated peroxidation. *Free Radic Biol Med* 2000;29:131–146.
- [22] Laureaux C, Thérond P, Bonnefont-Rousselot D, Troupel SE, Legrand A, Delattre J.  $\alpha$ -tocopherol enrichment of high-density lipoproteins: Stabilization of hydroperoxides produced during copper oxidation. *Free Radic Biol Med* 1997;22:185–194.
- [23] Havel RJ, Eder HA, Brandon JH. The distribution and chemical composition of centrifugally separated lipoproteins in human serum. *J Clin Invest* 1995;34:1345–1353.
- [24] Lai CS, Hopwood LE, Hyde JS, Lukiewicz S. ESR study of  $O_2$  uptake by Chinese hamster ovary cells during the cell cycle. *Proc Natl Acad Sci USA* 1982;79:1160–1170.
- [25] Thomas MJ, Chen Q, Zabalawi M, Anderson R, Wilson M, Weinberg R, Sorci-Thomas MG, Rudel LL. Is the oxidation of high-density lipoprotein lipids different than the oxidation of low-density lipoproteina lipids? *Biochemistry* 2001;40:1719–1724.
- [26] Buettner GR, Jurkiewicz BA. Catalytic metals, ascorbate and free radicals: Combination to avoid. *Radiat Res* 1996;145:532–541.