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Oxidation Induced Changes in Lipid Mobility in Lipoproteins Followed by Steady State Fluorescence Anisotropy

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The oxidation induced changes in the lipid layer of the lipoprotein particles of LDL and HDLs have been followed by measuring the steady-state polarization anisotropy of the fluorescence probe TMA-DPH incorporated in the lipid layer. The mobility restriction gradually increases with oxidation in LDL and HDL_2 indicating the oxidation-induced structuring of lipids, while the effect of oxidation in HDL_3 is negligible.

INTRODUCTION

All native serum lipoproteins have similar structures: the core of neutral lipids is encapsulated by the apolipoproteins embedded in a monolayer of polar lipids.[1] Although the »oil-drop« model has been well characterized, the molecular packing and lipid-protein interactions in the outer shell of various types of lipoprotein particles are still under investigation. Variations of lipid constituents in different lipoprotein classes induce differences in the molecular packing of the lipid monolayer as well as the specificity of apolipoproteins, thereby determining their role in lipid metabolism.[2]

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Since the discovery of the LDL receptor,[3,4,5] a close relationship between atherogenesis and lipid metabolism disorders has been established and intensively studied.[6,7] The main role has been ascribed to the disfunction of two lipoprotein classes: LDL and HDL. Based on numerous investigations, it has been accepted that oxidation of LDL is the main alteration of the lipoprotein particle promoting atherosclerosis.[8,9,10] Chemical changes in LDL induced by oxidation prevent its binding to the classical LDL receptor, but promote its recognition by scavenger receptors on macrophages. The transformation of the cell to lipid laden foam-cells is a hall-mark in the formation of atherogenetic lesions.[11,12] Esterbauer *et al.*[13,14] believe that the long-chain unsaturated fatty acid peroxidation is the initiating event, whereas protein modification is the secondary event.

The inverse relationship between the plasma HDL_2 concentration and the incidence of cardiovascular diseases pointed out its antiatherogenetic character.[15] HDL_2, modified by oxidation, loses its ability to stimulate an efflux of cholesterol from foam cells and loses its protecting function.[16]

The main, or most frequently reported, change in the surface structure of an oxidized lipoprotein particle is the measurable increase of the total negative charge.[17,18] Our previous extensive electron paramagnetic resonance studies on oxidised lipoproteins also indicated an enhanced number of binding sites for divalent cations[19] on the apolipoprotein B.

In the completely oxidized lipoprotein particles, the chemical changes in the lipid domain are associated with degradation of unsaturated fatty acids. Formation of aldehydes and lipid hydroperoxides[11,14,23] causes the increasing polarity of the lipid layer and a more ordered structure in the lipid tails.[24]

In the present study, the fluorescence intensity and steady-state anisotropy of the external fluorescence probe, incorporated into the phospholipid monolayer, were considered suitable quantities for monitoring the structural changes induced in LDL and HDL subfractions during the oxidation by Cu^{2+} ion.

EXPERIMENTAL

LDL, density 1.019–1.063 g/cm^3, and high density lipoprotein fractions HDL_2, density 1.085–1.125 g/cm^3, and HDL_3, density 1.125–1.21 g/cm^3, were isolated by sequential ultracentrifugation of fresh-drawn plasma from four healthy human donors carefully avoiding peroxidation by addition of 1 g/L of EDTA. The lipoproteins were dialyzed against a 10 mM sodium phosphate buffer, pH = 7.4, sterilized and used for oxidation within a month of isolation. They were oxidatively modified by incubation at 37 °C with $CuSO_4$ at a final concentration of 10 μM. The oxidation process was stopped at different time intervals by adding 1 mg/mL EDTA to the sample. The degree of oxidation was checked electrophoretically and by measuring the concentration of lipid hydroperoxide (LPO) formed from the unsaturated fatty acids.[20] The

concentration of the lipoproteins was determined according to Lowry, with bovine serum albumin as standard.[21]

Prior to fluorescence measurements, all samples were dialyzed against 0.1 M TRIS buffer containing 0.24 mM EDTA pH = 7.4. Lipid fluorophor, TMA-DPH, 1-(-4-trimethylammoniumphenyl)-6-phenyl-1,3,5,-hexatriene was incorporated into the lipid envelope of the lipoprotein particle directly before fluorescence measurements. The concentration of TMA-DPH was one molecule to approximately 250 lipid molecules. For this purpose, the ethanol stock solution of 1 mM TMA-DPH was directly added to the sample. The final ethanol concentration in the samples was less than 0.3%. For incorporation of the fluorescence label, the samples were slightly shaken for 20 minutes. The incorporation was assumed to be completed when the increasing fluorescence intensity of the label levelled out. The stability of the fluorescence label to the oxidation in oxidized lipoprotein was checked by measuring the time dependence of the fluorescence intensity in oxidized lipoproteins.

The fluorescence anisotropy was measured on a Hitachi F-4000 spectrofluorimeter equipped with a thermostatic cell unit. The desired temperature was kept constant at ±0.1 °C by water flow and the sample chamber was constantly flushed with nitrogen. The instrument was standardized with 0.1 μg/mL quinine sulphate in 0.1 N H_2SO_4. The band width was 10 nm for both excitation and emission and the wave lengths were 360 nm and 430 nm, respectively.

The steady-state fluorescence anisotropy, r_s, was determined by emission intensities through an analyzer oriented parallel, I_{II}, and perpendicular, I_I, to the direction of excitation light polarization

$$r_s = \frac{I_{II} - I_I}{I_{II} + 2\,I_I}$$

RESULTS

The oxidative modification of the lipoprotein particle was followed by monitoring the rate of lipid hydroperoxide production, as shown on Figure 1. The oxidation profile of LDL differs from the ones of HDL_2 and HDL_3 showing the well established three phase profile:[14] an initial lag-phase, a propagation phase and a decomposition phase. The oxidation curve of HDL_3 lacks the lag-phase, reflecting less resistance of this lipoprotein to oxidation and a much shorter time needed to achieve an oxidized state. The formation of LPO of HDL_2 increased shortly after the onset of oxidation, followed by a decrease and a second strong increase.

We wanted to find out whether the florescence anisotropy of TMA-DPH could be the parameter for following the coupling of the lipid core phase transition and the surface lipid motion. Therefore, the steady-state anisotropy variations of TMA-DPH, incorporated in native and oxidized LDL, with temperature were measured. The results are shown in Figure 2. The shapes of all curves, except for native LDL, are similar. The steady-state anisotropy of TMA-DPH incorporated in LDL of different oxidation states de-

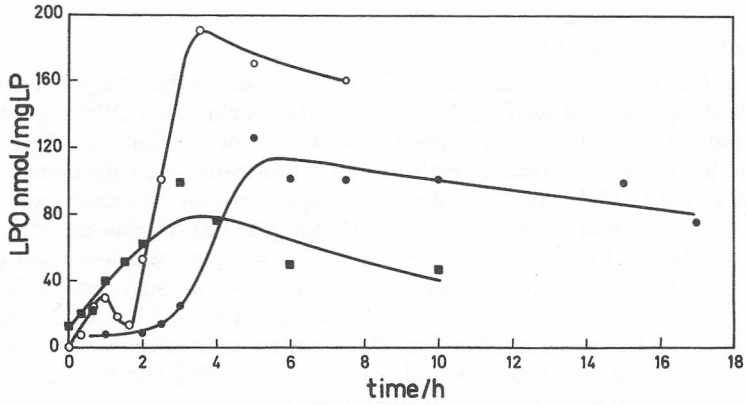

Figure 1. Time dependent oxidation of lipoproteins expressed as LPO concentration. Lipoprotein concentrations were 1.7 mg/mL for LDL (●), 1.5 mg/mL for HDL_2 (○) and 1.2 mg/ mL for HDL_3 (■).

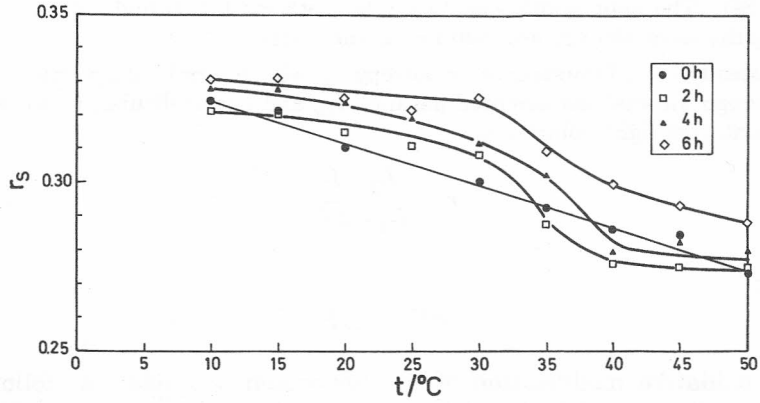

Figure 2. Temperature dependence of the steady-state anisotropy of TMA-DPH incorporated in gradually oxidized LDL. The wavelength of excitation, λ_{EX}, was 360 nm and emission, λ_{EM}, was 430 nm. The LDL concentration was 1.7 mg/mL.

creases with temperature. The slightly expressed threshold between 30 and 40 °C moves towards higher temperatures as the oxidation progresses. In all fluorescence measurements, the corresponding non-labelled but oxidized lipoproteins were used as blank.

Figure 3 presents the steady state anisotropy, r_s, in different oxidation stages for all three lipoproteins. The variations in anisotropy induced by oxidation in LDL resemble the three-stage oxidation curve shown in Figure 1. Disregarding the lag-phase existence, similar quantitative changes are detected upon oxidative modifications of HDL_2. For the probe incorporated in HDL_3, the detected changes are in the range of experimental error. The vari-

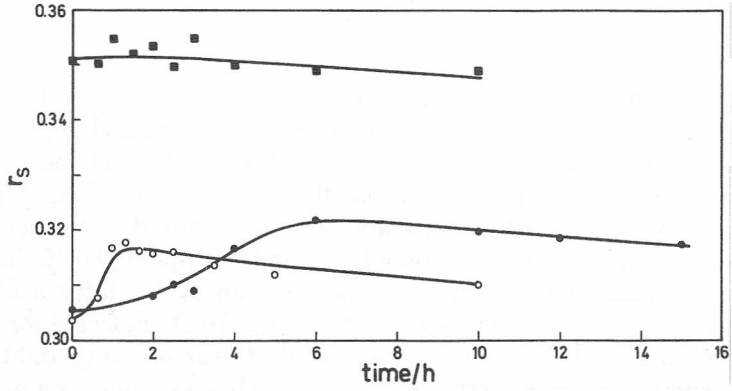

Figure 3. Dependence of steady-state anisotropy of TMA-DPH on the degree of oxidation for LDL (●), HDL_2 (○) and HDL_3 (■). The concentrations of lipoproteins are the same as in Figure 1. Measurements were performed at (24 ± 0.1) °C.

ation of r_s upon oxidation follows the order $HDL_3 < HDL_2 < LDL$ with the absolute values of $HDL_3 > HDL_2 \cong LDL$.

Prendergast *et al.*[21] have shown that the quantum yield of TMA-DPH in water is zero and that it increases with the apolarity of the media. Thus, the TMA-DPH fluorescence intensity can be used for sensing the polarity of its immediate vicinity. The measured intensity for all three lipoproteins, corrected for a small background due to the lipoprotein concentration and spectrometer condition, are presented in Figure 4. The fluorescence intensity increase upon oxidation is less pronounced in HDLs than in LDL. The lag-phase of the LDL oxidation is barely noticeable but, after reaching the maximum, the intensity decreases again while in HDL it reaches a limit value.

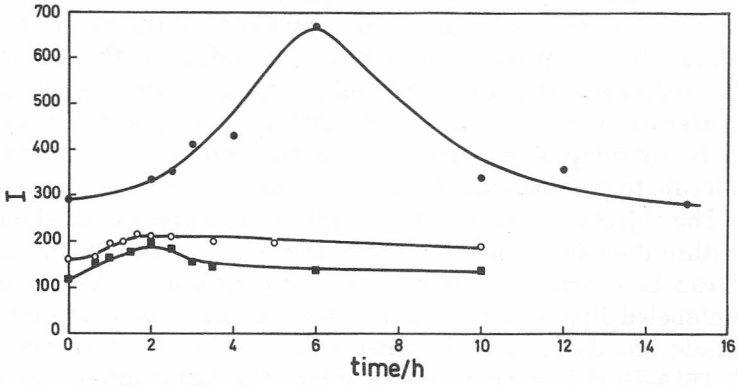

Figure 4. Dependence of the relative fluorescence intensity of TMA-DPH on the degree of oxidation of LDL (●), HDL_2 (○) and HDL_3 (■). The temperature and the lipoprotein concentration are the same as in Figure 3.

DISCUSSION

Our intention was to study the structural changes induced in the phospholipid monolayer by oxidative modification of two main lipoprotein fractions involved in atherosclerosis, LDL and HDLs. For that purpose, the lipoprotein particle was labelled with the fluorescent label, TMA-DPH. The fluorophor is by its trimethylammonium group anchored at the level of the polar heads of the phospholipid monolayer and the apolar DPH fluorescent moiety is intercalated between the upper portion of the fatty acid chains. Prendergast *et al.*[22] demonstrated that the TMA-DPH fluorescence parameters are sensitive to the polarity of the immediate surrounding and that they reflect the degree of order of the fatty acyl backbone region. The absorption and emission moments of the fluorophore are almost parallel and aligned to the longitudinal axis. Thus, not the rotation around the longitudinal axis but the wobbling motions around the axis that lies in the plane of the particle depolarize the emitted light. The extent of the depolarization reflects the degree to which a population of photo selected excited fluorophore loses its initial selective orientation and becomes randomized during the fluorescence life time. The restriction in the rotational motion of the fluorophore imposed by its immediate vicinity would be reflected in an increased steady-state anisotropy. So it would provide information about the fluidity of the layer. In general, it is difficult to evaluate the absolute motions of the system using a steady-state fluorescence depolarization technique; nevertheless, it provides a useful quantification of the effects of some perturbing events.

The increase in fluorescence intensities for the label in LDL has an intriguing shape (Figure 4). The peak in the curve correlates with the end of the propagation phase and the onset of the decomposition phase in particle oxidation. The increase of TMA-DPH fluorescence intensity during the propagation phase reflects the increasing apolarity of the media around the label, induced by the production of lipid peroxides. In the decomposition phase, the hydroperoxides are converted to reactive aldehydes of increasing polarity. Interaction of these aldehydes with the apolipoprotein residues diminishes the coupling of the apoprotein to the phospholipids[25] enabling the water molecule to approach the lipid layer, which causes a quenching of fluorescence. The objection that the fluorescent entities are produced on the apoprotein within the same range of fluorescence excitation and emission wavelengths[26] can be overruled. The measured intrinsic fluorescence intensities for the unlabeled lipoproteins (the results are not shown) are by an order of magnitude smaller and their contribution to the fluorescence intensity values of TMA-DPH behaviour is negligible. The lower intensities in native HDL particles, as compared to native LDL, mean that the probe is in more polar media. The smaller size of the particle and the higher protein to lipid ratio cause the probe to be more exposed to water surroundings. The oxida-

tion induced changes of fluorescence intensity in both HDL fractions are also smaller than in LDL, leading to the conclusion that the overall structure of the small particle is not dramatically changed by chemical disturbances induced by oxidation.

The restriction of lipid mobility induced upon oxidation follows the order LDL > HDL_2 > HDL_3. The absolute values of r_s for the label in lipids of LDL and HDL_2, (Figure 3), indicate a similar motional freedom and similar changes in the fluorophore ordering and consequently motional restriction. The substantial higher value of r_s in HDL_3 does not depend upon oxidation, reflecting slight mobility changes of the lipid label in the less mobile lipid environment. Our finding suggests that the lipid mobility restriction is dependent on the protein to surface ratio. The HDL_3 is the smallest particle with the highest protein to lipid ratio of the three lipoproteins. High r_s in native particle indicates the restricted motion of the probe, and this cannot be markedly influenced by oxidation. It has been argued that the state of lipid mobility is determined by the structure of apoprotein on the surface of the molecule.[2] The more surface spread apoprotein permits less mobility in the lipid layer. In this respect, our findings would support the overall accepted model of apoprotein spread onto the lipoprotein surface.[28]

An abrupt change in the anisotropy of TMA-DPH in LDL could be expected while passing the core transition temperature, assuming that the fluorophore anchored in the phospholipid monolayer would be sensitive to the mobility state of the core. Only a slightly marked threshold in the constantly decreasing anisotropy upon heating can be rationalized by, in the time scale of fluorescence measurements, the motional state of surface lipids being only weakly coupled with the state of lipids in the core. This is in agreement with Kroon's interpretation of the results on reconstituted LDL.[27] Of more value for our study is the ratio r_{50}/r_{10}, (Figure 2) as the parameter that reflects the increase in the mobility of the probe, or the looseness of the structural order, upon heating. The ratio of 1.3 for native LDL in comparison to 1.1 for highly oxidized LDL indicates a more efficient temperature induced increase in mobility in the native than in highly oxidized LDL. This means that the induced ordering of the phospholipid tails by oxidation and the consequent reduction of wobbling motions is barely changeable by thermal energy.

We have shown that the changes of fluorescence parameters of TMA-DPH by oxidation of lipoprotein could be conclusively interpreted as the oxidation polarizing the phospholipid interior and inducing more rigid structure into the phospholipid tails. At the same time oxidation diminishes the apoprotein phospholipid interactions, allowing the water molecules to approach the lipid domain of the particle.

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REFERENCES

1. D. Atkinson and D. M. Small, *Annu. Rev. Biophys. Chem.* **15** (1986) 403–456.
2. J. A. Ibdah, S. Lund-Katz, and M. C. Philips, *Biochemistry* **28** (1989) 1126–1133.
3. J. L. Goldstein and M. S. Brown, *J. Clin Invest.* **70** (1982) 1184–1192.
4. M. S. Brown and J. L. Goldstein, *Science* **232** (1986) 334–347.
5. M. S. Brown and J. L. Goldstein, *Nature* **343** (1990) 508–509.
6. J. W. Heinecke, *Free. Rad. Biol. Med.* **3** (1987) 64–73.
7. G. Jürgens, *Anu. Rep. Med. Chem.* **25** (1990) 169–176.
8. J. L. Witztum and D. Steinberg, *J. Clin. Invest.* **88** (1991) 1785–1792.
9. G. Jürgens, Q. Chen, G. Ledinski, G. Kager, A. Hammer, and H. Esterbauer, *AMA* **4** (1993) 85–89.
10. G. Jürgens, H. F. Hoff, G. M. Chisolm III, and H. Esterbauer, *Chem. Phys. Lipids* **45** (1987) 315–336.
11. H. Esterbauer, M. Dieber-Rotheneder, G.Waeg, G. Striegel, and G. Jürgens, *Chem. Res. Toxicol.* **3** (1990) 77–92.
12. U. P. Steinbrecher, *Curr. Opinion Lipidol.* **1** (1990) 411–415.
13. H. Esterbauer, G. Jürgens, O. Quehenberger, and E. Koller, *J. Lipid Res.* **28** (1987) 495–509.
14. H. Esterbauer, J. Gebicki, H. Puhl, and G. Jürgens, *Free Radical. Biol. Med.* **13** (1992) 341–390.
15. J. L. Breslow, *Proc. Nat. Acad. Sci. USA* **90** (1993) 8314–8318.
16. Y. Nagano, H. Arai, and T. Kita, *Proc. Nat. Acad. Sci. USA* **88** (1991) 6457–6461.
17. U. P. Steinbrecher, S. Parthasarathy, D. S. Leake, J. L. Witztum, and D. Steinberg, *Proc. Nat. Acad. Sci. USA* **81** (1984) 3883–3887.
18. J. W. Heinecke, H. Rosen, and A. Chait, *J. Clin. Invest.* **74** (1984) 1890–1894.
19. J. Brnjas-Kraljević, G. Pifat, J. N. Herak, and G. Jürgens, *Free. Rad. Res. Comms.* **14** (1991) 307–313.
20. M. El-Saadani, H. Esterbauer, M. El-Sayed, M. Goher, A. Y. Nasser, and G. Jürgens, *J. Lipid Res.* **30** (1989) 627–630.
21. O. H. Lowry, N. J. Rosenbrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.* **193** (1951) 265–275.
22. F. G. Prendergast, R. P. Haugland, and P. J. Callahan, *Biochemistry* **20** (1981) 7333–7338.
23. M. L. Lenz, H. Hughes, J. R. Mitchell, D. P. Via, J. R. Guyton, A. A. Taylor, A. M. Gotto Jr., and C. V. Smith, *J. Lipid Res.* **31** (1990) 1043–1050.
24. O. M. Panasenko, T. V. Vol'nova, O. A. Azizova, and Y. A. Vladimirov, *Free. Rad. Biol. Med.* **10** (1991) 137–148
25. S. Lund-Katz and M. C. Phillips, *Biochemistry* **25** (1986) 1562–1568.

26. L. Cominiacini, A. Garbin, R. Micciolo, O. Bosello, G. Gaviraghi, L. A. Scuro, and A. M. Pastorino, *J. Lipid Res.* **32** (1991) 349–358.
27. P. A. Kroon, *Biochemistry* **33** (1994) 4879–4884.
28. E. Schnitzer and D. Lichtenberg, *Chem. Phys. Lipids* **70** (1994) 63–74.

SAŽETAK

Promjene u gibljivosti lipida u oksidiranim LDL, HDL_2 i HDL_3 česticama promatrane mjerenjima fluorescentne anizotropije

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Mjerenjem fluorescencijskih parametara fluorescentne probe TMA-DPH inkorporirane u lipidnu frakciju lipoproteinskih čestica LDL i HDL promatrane su strukturne promjene u lipidnom dijelu za vrijeme oksidacijskog procesa. U česticama LDL i HDL_2 postupno se smanjuje pokretljivost lipida što upućuje na strukturiranje fosfolipida procesom oksidacije. Vrijednosti mjerenih parametara u čestici HDL_3 pokazuju da je gibljivost lipida u toj čestici ograničena i da su promjene koje uzrokuje oksidacija zanemarive.