Free Rad. Res. Comms., Vol. 14, Nos. 5–6, pp. 307–313
Reprints available directly from the publisher
Photocopying permitted by license only

EPR EVIDENCE FOR THE OXIDATION-INDUCED FORMATION OF NEGATIVELY CHARGED SPECIES ON THE LOW-DENSITY LIPOPROTEIN SURFACE

J. BRNJAS-KRALJEVIĆ [1], G. PIFAT[2], J.N. HERAK[3]* and G. JÜRGENS[4]

[1]School of Medicine, [2]Ruđer Bošković Institute and [3]Faculty of Pharmacy and Biochemistry, University of Zagreb, P.O. Box 156, 41001 Zagreb, Croatia, Yugoslavia, and [4]Institut für Medizinische Biochemie, Karl Franzens Universität, Harrachgasse 21, A-8010 Graz, Austria

(Received July 16, 1990; in final form: October 2, 1990)

Oxidation-induced increase of the net negative charge on low-density lipoprotein was studied by electrophoretic mobility and by electron paramagnetic resonance. The negative-charge increase is associated not only with neutralization of the lysine residues of apoprotein B, but also with the exposition of the excessive negatively charged residues on the lipoprotein surface. The accumulation of the negatively charged residues is believed to be brought about by the conformational change of apoprotein B, triggered by neutralization of lysines and cleavage of peptide bonds. Alternatively, reactive oxygen species could also convert histidine to aspartic acid and proline to glutamic acid.

KEY WORDS: EPR, low-density lipoprotein, ion binding, lipoprotein oxidation, electrophoretic mobility.

INTRODUCTION

It has been demonstrated that oxidation induces changes of a number of physical, chemical and biological properties of low-density lipoprotein (LDL).[1,2] One of the easily noticeable oxidation-induced change is the increase of electrophoretic mobility.[3–5] In that respect, oxidation induces the same type of change on LDL as does acetylation. More important, both oxidation and acetylation modify LDL in the way that it is recognized by scavenger receptors on macrophages.[3,5–11] In the process of acetylation, positively charged lysine residues of apoprotein B are neutralized, and consequently the total negative charge of the LDL particle is increased. Neutralization and derivatization of lysines also take place upon oxidation.[12] In the present study we demonstrate that the oxidation-induced increase of the LDL net negative charge cannot be explained simply by neutralization of lysine positive charges. It will be shown that oxidation induces new negatively charged sites on the LDL surface, probably associated with the conformational change and cleavage of apolipoprotein B or with the generation of negatively charged amino acids by the direct attack of reactive oxygen on certain amino acids.[3]

*To whom the correspondence should be addressed: Prof. Dr. J.N. Herak, Faculty of Pharmacy and Biochemistry, University of Zagreb, P.O. Box 156, A. Kovačića 1, 41001 Zagreb, Yugoslavia.

MATERIALS AND METHODS

LDL was isolated from the fresh-drawn blood by ultracentrifugation, carefully avoiding lipid peroxidation,[13] and dialysed against the Tris-HCl buffer containing EDTA, pH 7.4. Prior to the oxidation EDTA was removed by extensive dialysis against the Tris-HCl buffer of selected ionic strength. Oxidation was performed by bubbling oxygen through the buffer outside the dialysis bag containing LDL.[14] After a selected time of such a mild oxidation the electrophoretic mobility of the sample was checked on agarose gel plates (Lipidophor system, Immuno A.G., Vienna). The path of the LDL band traversed in 80 min was determined densitometrically.

The samples with oxidation-induced mobility were examined for their capacity to bind divalent cations, Mn(II) ions, by EPR spectroscopy.[15,16] The Mn(II) ions were used as a structural probe to characterize changes induced by oxidation on the LDL surface. As previously described,[15]the binding of Mn(II) ions to LDL was determined from the difference in the observed amplitudes of the charactristic Mn(II) free EPR spectra in the presence and absence of native and oxidatively modified LDL in the solution (Figure 1). Further oxidation was prevented prior to the EPR measurements by dialysis of oxidized LDL against appropriate buffer containing butylated hydroxytoluene. The EPR spectra were recorded with a Varian E-109 spectrometer at a temperature of 37°C, keeping the temperature constant within 0.1°C to avoid misleading results caused by the strong temperature dependence of the Mn(II) ion EPR spectra.

RESULTS

The manganese binding to native (unmodified) LDL has been thoroughly studied and

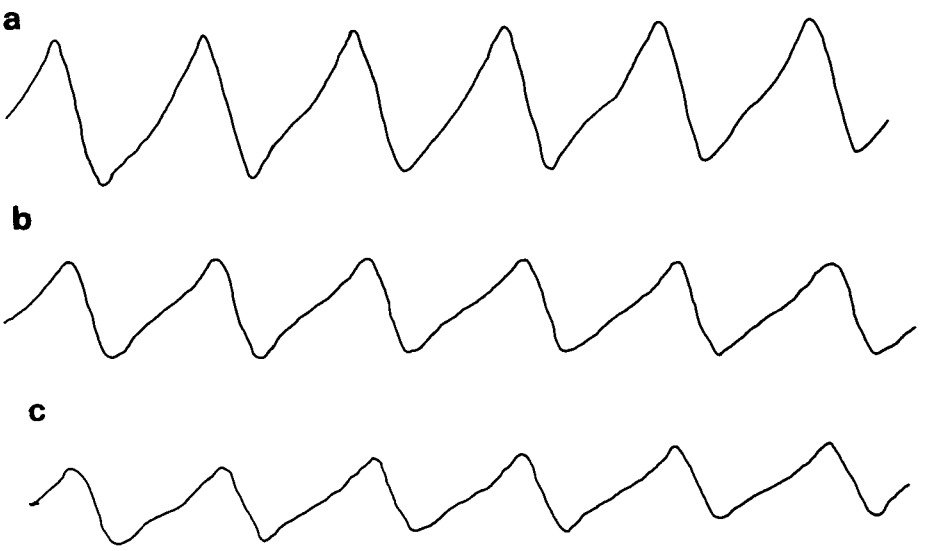

FIGURE 1 EPR spectra of 0.5 mM $MnCl_2$ in 0.02 M Tris-HCl, pH 7.4. A) without LDL; B) with 10.3 μmole native LDL/liter C) with 10.3 μmole oxidized LDL/liter.

the binding parameters rather precisely determined as a function of ionic strength.[16] Two types of independent binding sites have been found: n_1, "strong" binding sites characterized by a binding constant K_1, and n_2, "weak" binding sites with an association constant K_2. The "weak" sites are more pronounced in the buffer of high ionic strength, while the "strong" binding gives the main contribution to the LDL-ion binding when the LDL particles are in the low ionic buffer. The parameters of the manganese binding to native LDL have been determined by fitting the experimental data to the theoretical relation:

$$[\mathrm{Mn}]_T = [\mathrm{Mn}]_F \left[1 + \frac{K_1[n_1 LDL]_T}{1 + K_1[\mathrm{Mn}]_F} + \frac{K_2[n_2 LDL]_T}{1 + K_2[\mathrm{Mn}]_F} \right] \quad (1)$$

Expression (1) is derived from the simple binding scheme (2) and the definition of the association constants (3):

$$\mathrm{Mn} + nLDL \rightleftharpoons \mathrm{Mn}LDL$$

$$\begin{aligned} [\mathrm{Mn}]_T &= [\mathrm{Mn}]_F + [n_1 LDL]_B + [n_2 LDL]_B \\ [n_1 LDL]_T &= [n_1 \mathrm{LDL}]_F + [n_1 LDL]_B \\ [n_2 LDL]_T &= [n_2 LDL]_F + [n_2 LDL]_B \end{aligned} \quad (2)$$

$$K_1 = \frac{[n_1 LDL]_B}{[\mathrm{Mn}]_F [n_1 LDL]_F} \quad (3)$$

$$K_2 = \frac{[n_2 LDL]_B}{[\mathrm{Mn}]_F [n_2 LDL]_F}$$

where prefixes T, F and B to [Mn] denotes total, free and bound Mn(II) ion concentration, and to $[nLDL]$ total, free and bound concentration of binding sites on LDL surface, respectively.

These two types of binding sites in the native LDL surface are characterized by $n_1 = 8 \pm 3$ and $n_2 = 170 \pm 30$, with corresponding ionic-strength-dependent association constants.

Figure 2 shows the relative manganese binding $[\mathrm{Mn}]_B/[\mathrm{Mn}]_T$, to the oxidized *LDL*, in 0.02 M Tris-HCl buffer (experimental points). The figure also shows the binding curve (dashed line) of the native LDL under the same conditions (concentation, ionic strength and temperature). The curve has been calculated with the parameters ($n_1 = 8$, $n_2 = 170$, $K_1 = 4843\,\mathrm{M}^{-1}$ and $K_2 = 111\,\mathrm{M}^{-1}$) determinated in R.[16] It is obvious that the binding capacity of oxidized LDL is much higher than that of native LDL, particularly at low total Mn(II) concentration. To fit the experimental data for oxidized LDL to the theory, one or more of the four binding parameters (two binding constants, K_1 and K_2 and two numbers of binding sites, n_1 and n_2) has to be altered. The contribution of the abundant "weak" binding sites has been checked by Mn(II) binding to oxidized LDL in the buffer of higher ionic strength. At higher ionic strength (0.1 Tris-HCl) alterations of the binding parameters n_2 and K_2 are expected to be observed. From the measurements presented in Figure 3 it follows that these sites are not significantly affected by oxidation. In contrast to that, less abundant "strong" binding sites, represented by n_1 and K_1, expressed in the buffer of low ionic strength (Figure 2), manifest pronounced changes upon oxidation. The best fit of experimental points in Figure 2 has been achieved when only the value of n_1 has been

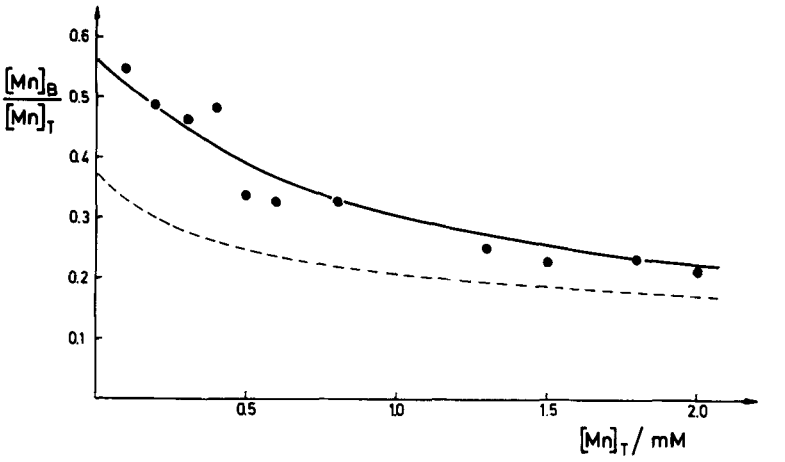

FIGURE 2 Binding of Mn(II) ions to LDL in 0.02 M Tris-HCl buffer. Relative binding of Mn(II) ions to LDL (10.3 μmole/liter, pH 7.4 at 37°C, in a 0.02 M Tris-HCl buffer) oxidized for 50 hours at room temperature. The dots are the experimental measurements and the solid line is the best fit to the relation (1), with the parameters $n_1 = 20$, $K_1 = 4843\,M^{-1}$, $n_2 = 170$, $K_2 = 111\,M^{-1}$. The dashed line represents the expected binding to native LDL.

changed. The measaurements presented in Figures 2 and 3 undoubtedly indicate the increase in number of "strong" binding sites. The best fit to the experimental points in both figures to the theoretical predictions (1) is obtained for the following set of binding parameters: $n_1 = 20 \pm 3$, $K_1 = 4843\,M^-$ for "strong" and $n_2 = 170 \pm 30$, $K_2 = 111\,M^{-1}$ for "weak" binding sites. These values differ only in n_1 (20 instead 8) from those for native LDL under same conditions. There is no evidence for the oxidation-induced change of the remaining three parameters.

A qualitatively very similar situation has been found in *LDL* samples oxidized for

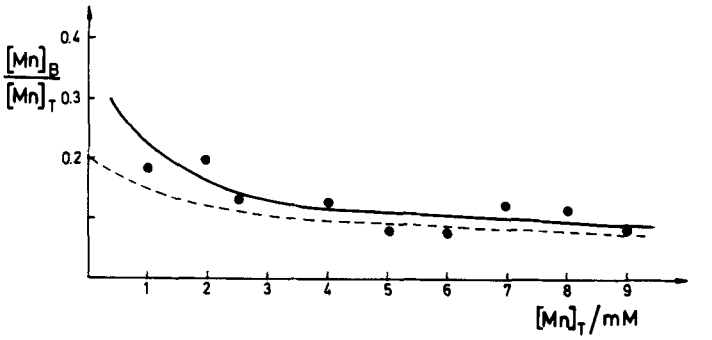

FIGURE 3 Mn(II) binding to LDL in 0.1 M Tris-HCl buffer. Relative binding of Mn(II) ions to LDL (10 μmole/liter, in a 0.1 M Tris-HCl buffer). The binding constants used for reconstruction of the binding curves (solid line and dashed line) are as those in Figure 2, corrected for the different electrolyte ionic strength ($K_1 = 746\,M^{-1}$, $K_2 = 64\,M^{-1}$). Other conditions are as in Figure 2.

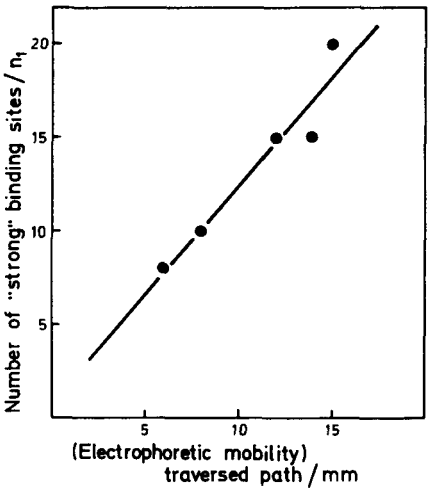

FIGURE 4 Number of Mn(II) binding sites vs. electrophoretic mobility. Correlation of electrophoretic mobility (measured as the total path traversed in 80 min.) and the number of "strong" binding sites, n_1, on the LDL surface.

shorter or longer times or in samples from five different preparations. To detect the increase in electrophoretic mobility the time of oxidation process depended on the *LDL* concentration and on the source of *LDL*. The increased electrophoretic mobility is associated with the increased number of "strong" binding sites, n_1, as shown in Figure 4 and is independent on *LDL* concentration or source of *LDL*. The increase from 8 (for unmodified *LDL*) to about 20 (for oxidized *LDL*) can be easily reached simply by mild oxygenation of the buffer. Stronger oxidation conditions (with 5–10 μM $CuCl_2$) lead to n_1 as large as 30.

DISCUSSION

Oxidation induces neutralization and derivatization of lysine residues in approtein B in *LDL*,[12] leading to an increase of the net negative charge and hence to an increase of the electrophoretic mobility of the LDL particle. In the present study, in addition to electrophoresis we have used the method (EPR) sensitive only to negatively charged species exposed on the lipoprotein surface which are responsible for the binding of paramagnetic Mn(II) ions. Comparison of the results obtained by the two methods demonstrates that the neutralization of lysine residues is accompanied by the appearance of additional binding sites with strong affinity for divalent cations on the *LDL* surface. Although the chemical nature of the binding sites is unknown, it is safely concluded that the protein residues bearing a net negative charge must be "strong", while it is speculated that the phospholipid head group on the *LDL* surface is "weak".[15]

The appearance of the oxidation-induced new negatively charged species associated with "neutralization" of lysine could be explained in terms of the conformational change of apoprotein B. It has already been shown that the breakdown products generated during the peroxidation of unsaturated fatty acids, such as malondial-

dehyde and 4-hydroxynonenal, are able to modify reactive amino groups of the protein moiety of *LDL*.[17-19] The neutralization of lysine might be a trigger for the conformational change if, for instance, positively charged lysine residues in native *LDL* are coupled with negatively charged residues, such as aspartic acid, glutamic acid or sialic acid. Alternatively, it has been reported that oxidation of *LDL* is accompanied by degradation of the apoprotein.[3] Such a degradation may lead to a completely new arrangement and embedding of shorter polypeptides on the *LDL* surface. However, the attack of reactive oxygen species could also directly generate new negatively charged amino acid residues. Thus, histidine could be converted to aspartic acid and proline to glutamic acid.[20]

The studies with monoclonal antibodies[21] support our findings and suggestions that newly created negative sites play a role in the interaction of oxidized *LDL* and its receptors.

Acknowledgements

This work was supported by the Yugoslav-American Joint Board for Scientific Cooperation, by the Croatian Self-Managing Scientific Community, the Yugoslav Federal Research Funds and the Austrian National Bank (Project No. 3696). We thank Mrs. B. Salzer for making the electrophoretic measurements.

References

1. G. Jürgens, H.F. Hoff, G.M. Chisolm III, and H. Esterbauer (1987) Modification of human serum low density lipoprotein by oxydation-characterization and pathophysiological implications. *Chemical and Physical Lipids*, **45**, 315–336.
2. D. Steinberg, S. Parthasarathy, T.E. Carew, J.C. Khoo and J.L. Witztum (1989) Beyond cholesterol, modifications of low-density lipoprotein that increase its atherogenicity. *New England Journal of Medicine*, **320**, 915–924.
3. U.P. Steinbrecher, S. Parthasarathy, D.S. Leake, J.L. Witztum and D. Steinberg (1984) Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proceedings of the National Academy of Science U.S.A.*, **81**, 3883–3887.
4. S. Parthasarathy, U.P. Steinbrecher, J. Barnett, J.L. Witztum and D. Steinberg (1985) Essential role of phospholipase A_2 activity in endothelial-cell induced modification of low density lipoprotein. *Proceedings of the National Academy of Science U.S.A.*, **82**, 3000–3004.
5. J.W. Heinecke, H. Rosen and A. Chait (1984) Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. *Journal of Clinical Investigation*, **74**, 1890–1894.
6. J.L. Goldstein, Y.K. Ho S.K. Basu and M.S. Brown (1979) Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producting massive cholesterol deposition. *Proceedings of the National Academy of Sciences U.S.A.*, **76**, 333–337.
7. R.W. Mahley, T.L. Innerarity, K.H. Weisgraber and S.Y. Oh (1978) Altered metabolism (in vivo and in vitro) of plasma lipoproteins after selective modification of lysine residues of the apoproteins. *Journal of Clinical Investigation*, **64**, 743–750.
8. T. Henriksen, E.M. Mahoney and D. Steinberg (1981) Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: recognition by receptors for acetylated low density lipoproteins. *Proceedings of the National Academy of Science U.S.A.*, **78**, 6499–6503.
9. M.S. Brown and J.L. Goldstein (1983) Lipoprotein metabolism in the macrophage: implications for cholesterol depositions in atherosclerosis. *Annual Reviews in Biochemistry*, **52**, 223–261.
10. H. Arai, T. Kita, M. Yokode, S. Naruimiya and C. Kawai (1989) Multiple receptors for modified low density lipoproteins in muscle endothelial macrophages: different uptake mechanisms for acetilated and oxidized low density lipoproteins. *Biochemical and Biophysical Research Communications*, **159**, 1375–1382.
11. C.P. Sparrow, S. Parthasarathy and D. Steinberg (1989) A macrophage receptor that recognizes

oxidized low density lipoprotein but not acetylated low density lipoprotein. *Journal of Biological Chemistry*, **264**, 2599–2604.
12. U.P. Steinbrecher (1987) Oxidation of human low density lipoproteins results in derivatization of lysine residues of apolipoprotein B by lipid peroxide decomposition products. *Journal of Biological Chemistry*, **262**, 3603–3608.
13. H. Esterbauer, G. Jürgens, O. Quehenberger and E. Koller (1987) Antioxidation of human low density lipoprotein: loss of polyunsaturated fatty acids and vitamin E and generation of aldehydes. *Journal of Lipid Research*, **28**, 495–509.
14. J. Schuh, G.F. Fairclough and R.H. Haschemeyer (1978) Oxygen-mediated heterogenity of apo-low-density lipoproteins. *Proceedings of the National Academy of Science U.S.A.*, **75**, 3173–3177.
15. J.N. Herak, G. Pifat, J. Brnjas-Kraljević and G. Jürgens (1982) Adsorption of Mn(II) ions to human low density lipoproteins. Magnetic resonance studies. *Biochimica et Biophysica Acta*, **710**, 324–331.
16. J.N. Herak, Lj. Udovičić, G. Pifat, J. Brnjas-Kraljević, G. Jürgens and A. Holasek (1988) An ESR study of the effect of an electrostatic field on binding of divalent cations to the surface of serum low-density lipoproteins. *Biochimica et Biophysica Acta*, **876**, 200–209.
17. M.E. Haberland, A.M. Fogelman and P.A. Edwards (1982) Specificity of receptor-mediated recognition of malondialdehyde-modified low density lipoproteins. *Proceedings at the National Academy and Science U.S.A.*, **79**, 1712–1716.
18. M.E. Haberland, C.L. Olch and A.M. Fogelman (1984) Role of lysines in mediating interaction of modified low density lipoproteins with the scavenger receptor human monocyte macrophages. *Journal of Biological Chemistry*, **259**, 11305–11311.
19. G. Jürgens, J. Lang and H. Esterbauer (1988) Modification of human low density lipoproteins by the lipid peroxidation product 4-hydroxy-nonenal. *Biochimica et Biophysica Acta*, **875**, 103–114.
20. E.R. Stateman (1989) Oxygen radical mediated damage of enzymes: biological implications in medical, biochemical and chemical aspects of free radicals. in: *Medicinal Biochemical and Chemical Aspects of Free Radicals*. Eds. Hayaishi, O., Niki, E., Kondo. M. and Yoshikawa, T. Elsevier Science Publishers B.V., Amsterdam, pp. 11–19.
21. Z. Zawadzki, R.W. Milne and Y.L. Marcel (1989) An immunochemical marker of low density lipoprotein oxidation. *Journal of Lipid Research*, **30**, 885–891.

Accepted by Prof. H. Sies