Free Rad. Res. Comms. Vol. 8, No. 3, pp. 175–183
Reprints available directly from the publisher
Photocopying permitted by license only

NMR ANALYSIS OF LOW-DENSITY LIPOPROTEIN OXIDATIVELY-MODIFIED *IN VITRO*

L. BARENGHI, S. BRADAMANTE,[1] G.A. GIUDICI and C. VERGANI[2]*

Fondazione Rivetti, Laboratorio di Biochimica e Biologia Molecolare, Viale Monte Nero 32 Milano, [1] CNR Centro Studio Sintesi e Stereochimica di Speciali Sistemi Organici, Universita' di Milano, Via Golgi 19 Milano, [2] Istituto di Medicina Interna, Universita' di Milano, Via Pace 9 Milano, Italy.

(Received September 8th, 1989; in revised form October 4th, 1989)

Human plasma low density lipoprotein has been oxidized at different stages *in vitro* and analysed by ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy and by biochemical methods. Information was obtained on: a) structure mobilities of lipids and on lipid-protein interactions; b) conjugated and oxo-dienes; c) polyunsaturated/monounsaturated fatty acid chains; d) lysophosphatidylcholine production. The results show that the NMR approach is particularly useful for the assessment of structural modification in oxidized LDL.

KEY WORDS: lipid peroxidation, NMR spectroscopy, oxidatively-modified LDL, atherosclerosis, ageing.

1. INTRODUCTION

Atherosclerosis is considered at present to result from endothelial injury caused by different starting agents.[1] Lipid accumulation (mainly cholesterol esters derived from LDL) and atheromatous plaques are formed after repeated endothelial injuries. Multiple mechanisms might take part in the formation of plaques; several reports have shown that altered lipoproteins may play an important role in foam cell formation.[2] Oxidatively-modified low density lipoprotein (ox-LDL) *in vivo* is potentially atherogenic because of an altered interaction with the LDL receptor, massive deposition of cholesterol esters in macrophages mediated by the LDL scavenger receptor, and cytotoxicity.[3,4] Until now only indirect and inconclusive evidence has been presented that ox-LDL occurs *in vivo* and is of any meaning in atherosclerosis.[2] Nevertheless modified lipoproteins have been found in atheromatous plaques[5,6] and in plasma of normolipidaemic subjects.[7] Rapid catabolism of ox-LDL,[8] oxidation product instability, and/or inhibition of extensive oxidation exerted by plasma antioxidants may be the cause of low circulating levels of mild ox-LDL.[4] Alternatively, the low levels of circulating ox-LDL may be due to the low sensitivity of the analytical procedures applied to determine lipid peroxidation in biological samples[9–12] or even

Abbreviations: LDL, low density lipoprotein; PC, phosphatidylcholine; lyso-PC, lysophosphatidylcholine; FA, fatty acid; PA_2, phospholipase A_2; DC, diene-conjugated lipid; OXO-D, oxo-diene; TNBS, trinitrobenzenesulphonic acid; TBARS, thiobarbituric acid-reactive substance(s); LPO, lipid hydroperoxides; BHT, butylated hydroxytoluene; TMS, tetramethylsilane; MDPA, methylemediphosphonic acid; SM, sphingomyelin.

* To whom correspondence should be addressed.

to the loss of water-soluble lipid peroxides during LDL isolation. We applied ^{1}H, ^{13}C, and ^{31}P NMR spectroscopy to study ox-LDL. Advantages over other techniques include the quite rapid, non-destructive procedure, the chance to study several lipid compounds at the molecular level, and to get evidence on the structural mobilities and interactions of the lipid-protein complexes.[13] Here we report results about *in vitro* ox-LDL, the model we have chosen to test the specificity and the sensitivity of the NMR approach.

2. MATERIALS AND METHODS

2.1. Isolation, Modification and Characterization of LDL

Human LDL (density 1.019–1.063 g/mL) samples were isolated by ultracentrifugation[14] from normolipidaemic pooled plasma collected in dipotassium EDTA (1.7 mg/mL). LDL was dialyzed against phosphate buffer (0.1 M, pH 7.4) containing 150 mM NaCl and 10 μM EDTA-Na_2 for 24 hours at 4°C. LDL was sterilized by filtration (0.2 μm Nalgene filter) and *in vitro* oxidation was obtained at 37°C by adding different amounts of metal ion according to the following procedures: i) LDL (diluted up to 31–34 mg total cholesterol/dL with EDTA-free dialysis buffer) was incubated with 5 μM $CuSO_4$ for 29 h as reported by Steinbrecher;[8] ii) alternatively LDL (230–260 mg total cholesterol/dL in dialysis buffer) was incubated with $Fe(NH_4)_2(SO_4)_2$ and $FeCl_3$ (400 μM each final concentration) for 68 h. Control incubations (c-LDL) were done in the presence of 200 μM EDTA-Na_2 without metal ions. In both cases the oxidation was stopped by refrigeration with the addition of 1 mM EDTA-Na_2 and 40 μM BHT. All samples were dialyzed at 4°C against 1 mM EDTA-Na_2, 150 mM NaCl in H_2O (pH 7.4) for 24 h and against 1 mM EDTA-Na_2 in D_2O (pH 7.4) for 12 h. TBARS were determined by using a modified method of Morel[15,16] in the unfractionated medium at the end of oxidation. At the end of the last dialysis, LPO was measured as described by Ohishi *et al.*[17] using a commercial test kit (Determiner LPO, Kyowa Medex Co, Japan). Protein was assayed by the method of Lowry.[18] Free amino group change was determined by testing the TNBS reactivity.[19] We used enzymatic kits according to the manufacturer's instructions to determine: a) total cholesterol and triglycerides (Boehringer Biochemica Robin); b) free cholesterol and total phospholipids (Biotrol). We quantified DC and OXO-D[20] on LDL extracts.[21] Fe or Cu concentration was determined in the ox-LDL fractions by inductively-coupled atomic emission spectroscopy.[22] *Cis*-9, *trans*-11-tetradecadienol was bought from Sigma, and butadiene monoxide from Aldrich.

2.2. NMR Spectroscopy

High resolution ^{1}H, ^{13}C, ^{31}P spectra have been recorded on a Varian XL-300 instrucumulated using a normal single pulse sequence and chemical shifts were referred to TMS as external standard. When necessary the H_2O signal was suppressed either by a continuous secondary irradiation or by a 3.5 s presaturation pulse at the H_2O resonance frequency. ^{31}P spectra were recorded at 121.50 MHz using a normal single pulse sequence with complete decoupling of protons; a delay of 7 s was used to allow recovery with a 90 pulse of 18 μs; shifts were referred to 85% H_3PO_4 as external standard.

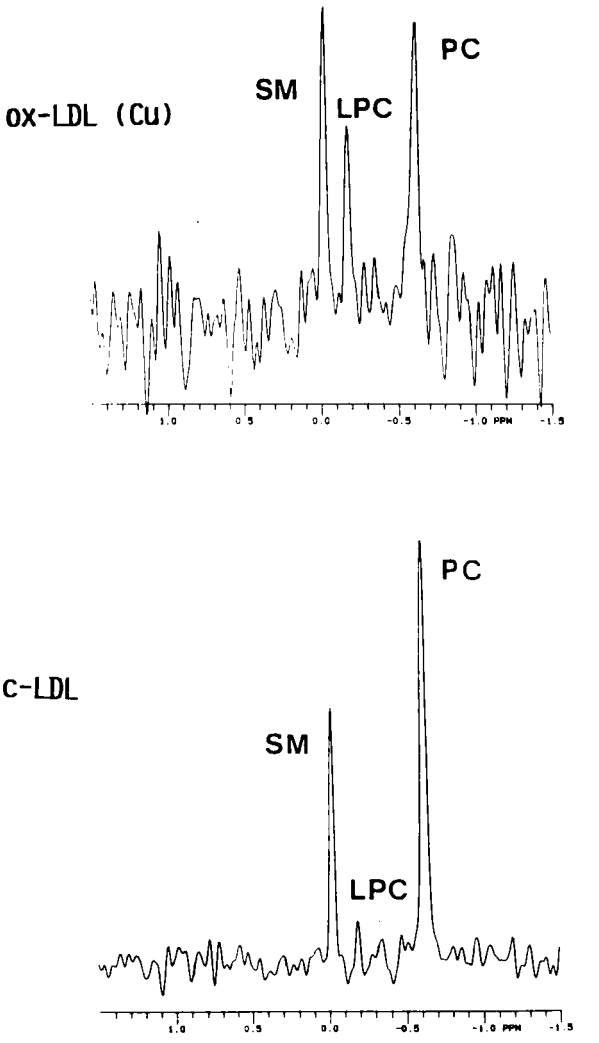

FIGURE 1 ^{31}P NMR spectra of c-LDL and copper ox-LDL (procedure i).

3. RESULTS

We chose a free radical-mediated process catalyzed by transition metals (copper and iron salts) to produce *in vitro* ox-LDL. Modifications were monitored by altered lipid composition, oxidation marker increase (such as TBARS, DC, OXO-D and LPO concentration) and decrease of free amino groups as reported in Table 1. We found 1 μM copper concentration in reisolated LDL prepared according procedure (i). We applied ^{31}P NMR analysis to quantitate the different phospholipids in LDL. In fact, they can be dispersed in mixed micelles by addition of sodium cholate[23] and compared to MDPA standard. ^{31}P spectra and quantitative analysis of ox-LDL and c-LDL

TABLE 1
Biochemical Analysis of c-LDL and ox-LDL

Sample	TC (a)	FC (a)	TG (a)	TBARS (b)	DC (c)	OXO-D (c)	LPO (g)	Free amino groups (d)	PL (e)	Lyso-PC/PC (f)
Procedure (i)										
c-LDL	1.66	0.42	0.30	n.d.			7.83	0.80	1.30	$\leqslant 0.05$
	(0.13)	(0.18)	(0.06)				(0.34)	(0.08)	(0.10)	
ox-LDL	1.08	0.38	0.23	22.66	231	146	246.00	0.28	1.21	0.66
	(0.13)	(0.05)	(0.03)	(2.86)			(14.04)	(0.05)	(0.13)	
Procedure (ii)										
c-LDL	1.53	0.48	0.21	0.88			22.9	0.73	1.13	$\leqslant 0.05$
	(0.22)	(0.06)	(0.06)	(0.08)			(3.5)	(0.06)	(0.03)	
ox-LDL	1.46	0.45	0.24	2.36	48	79	71.9	0.78	1.05	0.09
	(0.14)	(0.02)	(0.08)	(0.12)			(18.6)	(0.08)	(0.13)	

c-LDL and ox-LDL prepared following procedure (i) and (ii) were analysed as described in Methods (TC = total cholesterol; FC = free cholesterol; TG = triglyceride; PL = total phospholipid; TBARS, DC, LPO, Lyso-PC, PC as reported in abbreviations). Each result is the mean ($\pm$SD) of three experiments.

n.d. = not determinable
a = mg lipid/mg protein
b = nmol MDA/mg protein
c = μmol/mmol total phospholipids % increase
d = nmol/ug protein
e = μmol/mg protein
f = molar ratio
g = μmol/mmol total phospholipids

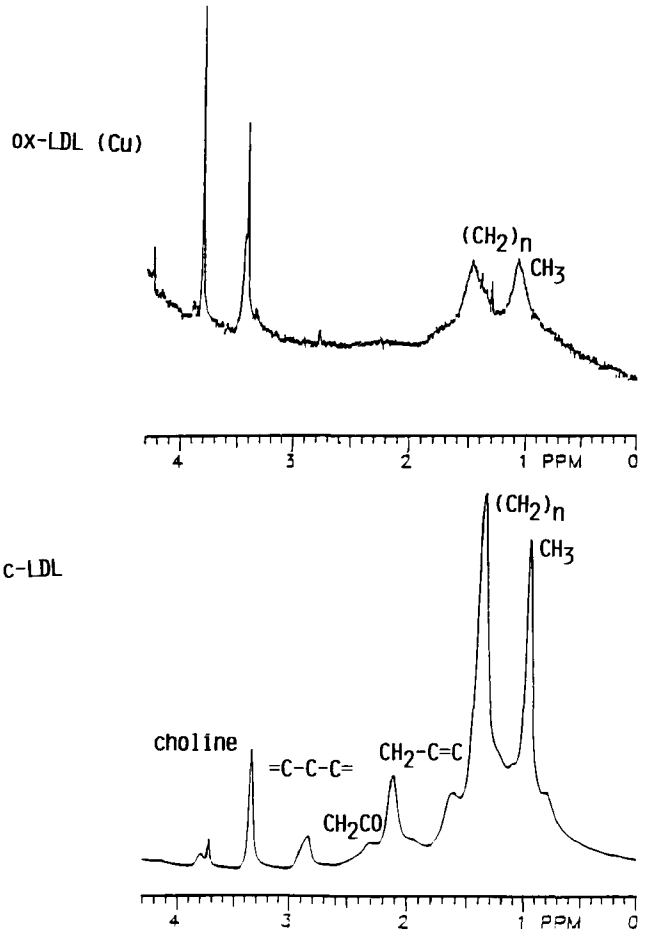

FIGURE 2 ^{1}H NMR spectra of c-LDL and copper ox-LDL (procedure i).

prepared according procedure (i) are reported in Figure 1 and Table 1. Differently from what we expected ox-LDL ^{1}H spectra at 37°C (Figure 2) showed very little evidence of peaks attributed to the fatty chain (2.5–0.5 ppm) and to the cholesterol moiety: we obtained no marked improvement by changing the temperature (45°C) or the accumulation time (nt = 5000). We run NMR spectra of ox-LDL prepared following procedure (ii). To overcome possible copper interference on ^{1}H spectra we used an iron salt ratio known to promote oxidation.[24] Despite the slight changes in lipid composition, in reactive amino group amount, and the very low TBARS of the ox-LDL (Table 1) we could check the oxidized status by NMR analysis. In this case the ^{13}C NMR spectra (Figure 3) revealed a change in the olefinic region (120–140 ppm). The ratio of the peak at 128.0 ppm (polyunsaturated carbons) to that at 129.7 ppm (monounsaturated carbons) is appreciably different, being above 1.1 in c-LDL and below 0.90 in ox-LDL. In addition several patterns appeared in the range 131–140 ppm, that we preliminarily attributed to conjugated dienes. ^{31}P NMR data

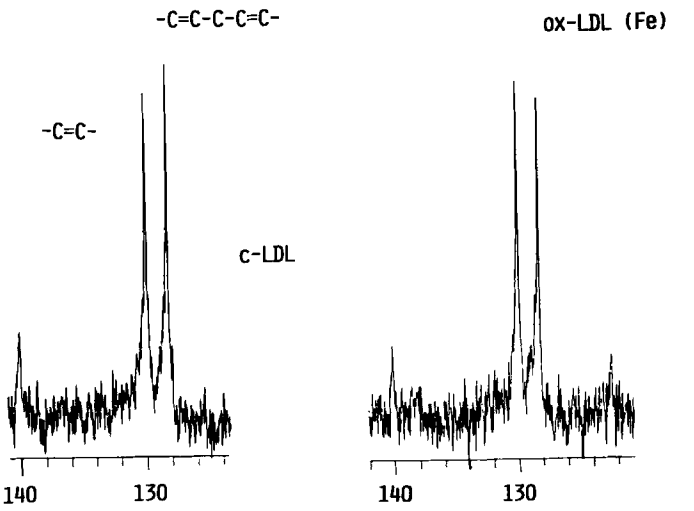

FIGURE 3 ^{13}C NMR spectra of c-LDL and iron ox-LDL (procedure ii).

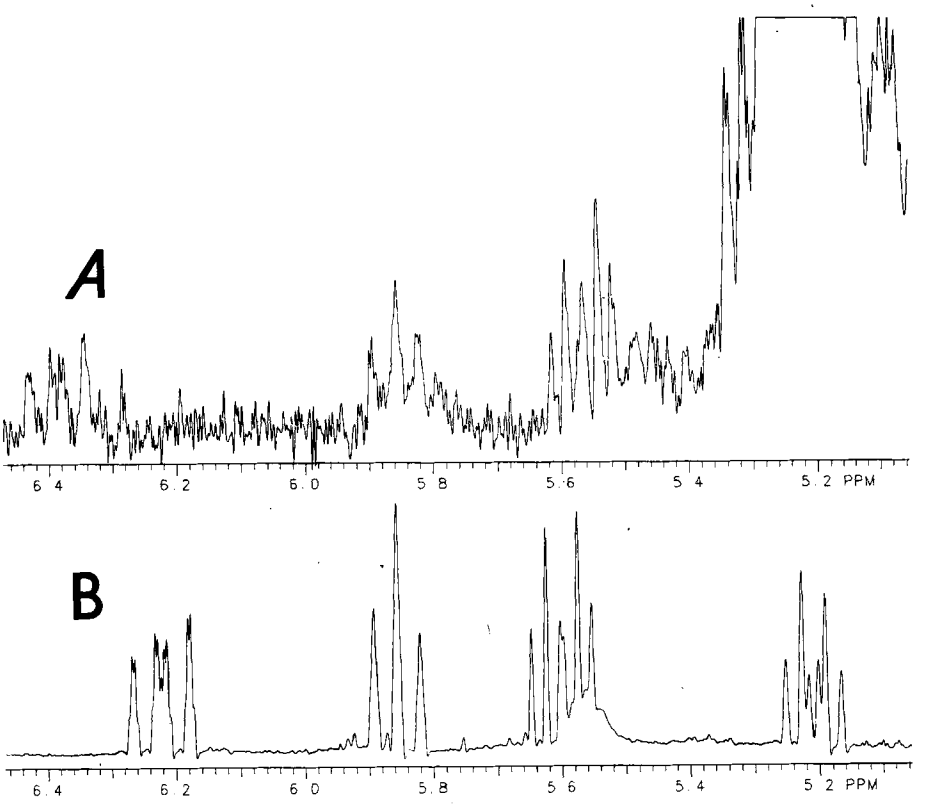

FIGURE 4 ^{1}H NMR spectra ($CDCl_3/CD_3OD$ 2/1 v/v) of (A) an iron ox-LDL extract (procedure ii), and (b) *cis*-9, *trans*-11-tetradecandienol.

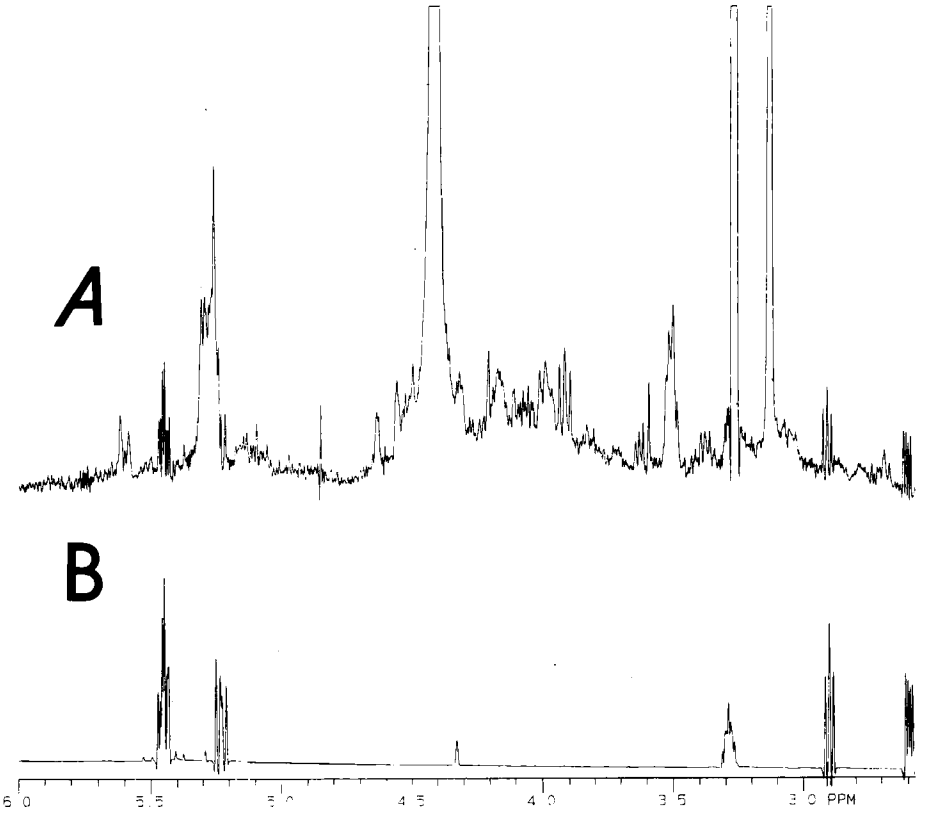

FIGURE 5 ^{1}H NMR spectra ($CDCl_3/CD_3OD$ 2/1 v/v) of (A) a copper ox-LDL extract (procedure i), and (B) butadiene monoxide.

showed a slight increase of lyso-PC (0–5%) in ox-LDL compared to c-LDL (Table 1). The ^{1}H spectra were similar to those obtained from c-LDL showing that this procedure induced no or very little modifications of the lipoprotein structure. We proved the presence of dienes analysing the spectra of ox-LDL extracts. The olefinic patterns have been attributed to a part of the *cis-trans* diene system (5.4–6.4 ppm) in comparison with the spectrum of *cis*-9, *trans*-11 tetradecadienol. Details are reported in Figure 4. Peaks of ox-LDL have been assigned as following: 6.39 ppm (H_1, dd) J_{trans} = 16 Hz, $J_{H_1H_2}$ = 9 Hz; 5.86 ppm (H_2,t) J_{cis} = 9 Hz, $J_{H_1H_2}$ = 9 Hz; 5.56 ppm (H_3,dt) J_{trans} = 16 Hz, $J_{H_3H_2}$ = 6.5 Hz. Instead, the ^{1}H NMR spectra of ox-LDL extracts (procedure i) showed a large amount of OXO-D residues, as the major result of the oxidation process. We identified this system by comparing the patterns with those obtained by NMR analysis of butadiene monoxide (Figure 5). Peaks of ox-LDL were attributed as follows: 5.45 ppm, CH=; 2.9 and 2.6 ppm epoxy system. NMR data are consistent with DC, OXO-D and LPO values from biochemical analysis.

4. DISCUSSION

We attribute ^{1}H NMR spectral changes of extensively ox-LDL (procedure i) to many concurrent causes such as: 1) the small paramagnetic effect of the copper, which remains bound to Apo B[3] even after extensive dialysis. 2) The LDL structural rigidity caused by bonds between aldehydes and protein and lipids[4] – in our experiments only 35% of free amino groups in ox-LDL were seen compared to control. 3) The increased density of ox-LDL due to an altered lipid/protein ratio.[8] Extensive loss of lipids in ox-LDL is in accord with Steinbrecher data.[8] 4) The decreased fluidity and increased order of the phospholipid acyl chains[25] and/or the change of the melting point caused by oxidized lipid products.[26] 5) The influence of the increased lyso-PC content on ^{1}H spectra as suggested by Steim.[27] Our data on phospholipid concentration are compatible with an extensive hydrolysis of PC by phospholipase A_2-like activity[8] due to oxidation. Therefore we suggest that the extensively ox-LDL (procedure i) could not be considered representative of the ox-LDL present *in vivo*. The ox-LDL in atheromatous plaques and in blood obtained from WHHL rabbits contained not more than 15 and less than 1 nmol MDA/mg protein respectively.[6] Human complicated plaques have been reported to contain a considerable amount of cholesterol esters of 9- and 13-hydroxyoctadeca-dienoic acids.[28] Indeed, there is no evidence that extensively ox-LDL might occur in human plasma.[2,7] Moreover a mild peroxidative premodification of LDL in the circulation might increase the deeper modifications in the vessel wall[29] and promote atherosclerosis. Ox-LDL obtained by using procedure (ii) appeared only mildly oxidized because of the low levels of TBARS concomitant with no changes in TNBS reactivity. ^{31}P NMR spectra revealed a slight increase in lyso-PC/PC ratio in ox-LDL compared to c-LDL showing a mild phospholipase A_2 activation consistent with low LPO and DC amounts. Nevertheless the olefinic region of ^{13}C NMR spectra revealed a change in polyunsaturated/monounsaturated fatty chains. ^{1}H NMR spectra of mild ox-LDL extracts (procedure ii) confirm the presence of DC mixture of isomers mainly *cis-trans* oriented. Extensively ox-LDL (procedure i) extracts revealed the presence of OXO-D possibly derived from the reaction of DC with hydroxyl radicals.[30] NMR analysis of our models is informative of the stages of lipid peroxidation on LDL *in vitro*. These data suggest that the NMR approach is a suitable device to study mildly oxidized LDL which might play a role *in vivo* during atherogenesis and the ageing process. Studies are in progress to compare our models with biological samples as whole plasma, plaques, and lipoproteins obtained from aged and high cardiovascular risk subjects.

References

1. Ross, R. The pathogenesis of atherosclerosis – an update. *New Engl. J. Med.*, **314,** 488–500, (1986).
2. Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C., and Witztum, J.L. Beyond cholesterol. Modifications of low density lipoprotein that increase its atherogenicity, *New Engl. J. Med.*, **320,** 915–924, (1989).
3. Jurgens, G., Hoff, H.F., Chisolm III, G.M., and Esterbauer, H. Modification of human serum low density lipoprotein by oxidation – Characterization and pathophysiological implications, *Chem. Phys. Lipids*, **45,** 315–336, (1987).
4. Heinecke, J.W. Free radical modification of low density lipoprotein: mechanism and biological consequences, *J. Free Radical Biol. Med.*, **3,** 65–73, (1987).
5. Shaikh, M., Martini, S., Quiney, J.R., Baskerville, P., La Ville, A.E., Browse, N.L., Duffield, R., Turner, P.R., and Lewis, B. Modified plasma-derived lipoproteins in human atherosclerotic plaques, *Atherosclerosis*, **69,** 165–172, (1988).

6. Daugherty, A., Zweifel, B.S., Sobel, B.E., and Schonfeld, G. Isolation of low density lipoprotein from atherosclerotic vascular tissue of Watanabe Heritable Hyperlipidemic Rabbits, *Arteriosclerosis*, **8,** 768–777, (1988).
7. Avogaro, P., Bittolo Bon, G., and Cazzolato, G. Presence of a modified low density lipoprotein in humans, *Arteriosclerosis*, **8,** 79–87, (1988).
8. Steinbrecher, U.P., Witztum, J.L., Parthasarathy, S. and Steinberg, D. Decrease in reactive amino groups during oxidation or endothelial cell modification of LDL, *Arteriosclerosis*, **7,** 135–143, (1987).
9. Gutteridge, J.M.C., Westermarck, T. and Halliwell, B. Oxygen radical damage in biological system, in: Free Radicals, Aging, and Degenerative Diseases, pp. 99–139, Alan R. Liss, Inc., (1985).
10. Knight, J.A., Pieper, R.K., and McClelian, L. Specificity of the thiobarbituric acid reaction: its use in studies of lipid peroxidation, *Clin. Chem.*, **34,** 2433–2438, (1988).
11. Esterbauer, H., Jurgens, G., Quehenberger, O., Koller, E. Autoxidation of human low density lipoprotein: loss of polyunsaturated fatty acids and vitamin E and generation of aldehydes, *J. Lipid Res.*, **28,** 495–509, (1987).
12. Matsushita, S., Terao, J. and Shibata, S.S. Limitations of the hemoglobin method for detecting lipid hydroperoxides, *J. Free Radical Biol. Med.*, **3,** 335–336, (1987).
13. Hamilton, J.A. and Morrisett, J.D. Nuclear magnetic resonance studies of lipoproteins, *Methods Enzymol.*, **128,** 472–515, (1986).
14. Havel, R.J., Eder, H.A. and Bragdon, J.H. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum, *J. Clin. Invest.*, **43,** 1345–1353, (1955).
15. Morel, D.W., Hessler, J.R., and Chisolm, G.M. Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipid, *J. Lipid Res.*, **24,** 1070–1076, (1983).
16. Jain, S.K. Membrane lipid peroxidation in erythrocytes of the newborn, *Clin. Chim. Acta*, **161,** 301–306, (1986).
17. Ohishi, N., Ohkawa, H., Miike, A., Tatano, T. and Yagi, K. A new assay method for lipid peroxides using a methylene blue derivative, *Biochem. Int.*, **10,** 205–211, (1985).
18. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, **193,** 265–275, (1951).
19. Steinbrecher, U.P. Oxidation of human low density lipoprotein results in derivatization of lysine residues of apolipoprotein B by lipid peroxide decomposition products, *J. Biol. Chem.*, **262,** 8, 3603–3608, (1987).
20. Kim, R.S. and LaBella, F.S. Comparison of analytical methods for monitoring autoxidation profiles of authentic lipids, *J. Lipid Res.*, **28,** 1110–1117, (1987).
21. Perkins, E.G. in: Analysis of Lipids and lipoproteins. pp. 1–23. The American Oil Chemists' Society, Champaign, Illinois, (1975).
22. Tietz, N.W. in: Textbook of Clinical Chemistry, pp. 984–985 and 1583, (1986).
23. Bradamante, S., Barchiesi, E., Barenghi, L., Zoppi, F. An alternative expeditious analysis of phospholipid composition in human blood plasma by P-31 NMR spectroscopy, *Anal. Biochem.*, in press.
24. Braughler, J.M., Duncan, L.A., Chase, R.L. The involvement of iron in lipid peroxidation, *J. Biol. Chem.*, **261,** 22, 10282–10289, (1986).
25. Eichenberger, K., Bohni, P., Winterhalter, K.H., Kawato, S. and Richter, C. Microsomal lipid peroxidation causes an increase in the order of the membrane lipid domain, *FEBS Lett.*, **142,** 59–62, (1982).
26. Dormandy, T.L. and Wickens, D.G. The experimental and clinical pathology of diene conjugation, *Chem. Phys. Lipids*, **45,** 353–364, (1987).
27. Steim, J.M., Edner, O.J. and Bargoot, F.G. Structure of human serum lipoproteins: nuclear magnetic resonance supports a micellar model, *Science*, **162,** 909–911, (1968).
28. Harland, W.A., Gilbert, J.D., Steel, G. and Brooks, C.J.W. Lipids of human atheroma, *Atherosclerosis*, **13,** 239–246, (1971).
29. Klimow, A.N. Formation in an animal organism of modified lipoproteins with an increased affinity for macrophages receptors, *International Atherosclerosis Congress, 20–22 May, Vienna.* p. 42 (1989).
30. Frankel, E.N. Secondary products of lipid oxidation, *Chem. Phys. Lipids*, **44,** 73–85, (1987).

Accepted by Prof. B. Halliwell