

Desialylation of Low Density Lipoprotein – Metabolic Function versus Oxidative Damage?

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Low Density Lipoprotein (LDL), N-acetyl-neuraminic-acid, Copper, Oxidation

Low density lipoproteins are generally considered to play a major role in the development of atherosclerotic vascular diseases. There is growing interest in LDL subspecies, especially in their density, carbohydrate content and oxidizability, which is supposed to enhance atherogenicity. We investigated the influence of desialylation on the resistance of the lipoprotein particles towards Cu(II) prooxidative activity.

Introduction

Low density lipoprotein (LDL) is the major transport form of cholesterol and cholesteryl esters in human plasma. It represents a highly compartmented system consisting of a lipid core mainly including cholesterol and phospholipid molecules as well as lipophilic antioxidants and one large protein moiety, the apolipoprotein B-100 (apoB-100). Oxidative modification by cells (macrophages, endothelial cells, smooth muscle cells), enzymes (lipoxygenase) or transition metal ions (Cu^{2+}) leads to lipid peroxidation and fragmentation of the apolipoprotein and renders the particle highly atherogenic causing foam cell formation of macrophages and lipid accumulation in the intima of the arterial wall. The uncontrolled uptake of oxidized LDL by macrophages through their scavenger receptor seems to be an important step in the development of atherosclerotic plaques (Brown and Goldstein, 1985; Parthasarathy and Rankin, 1992).

N-acetyl-neuraminic-acid (NANA) plays a particular role in receptor-mediated processes. NANA terminates complex carbohydrate chains in glycoproteins and glycolipids. It is removed by the action of neuraminidase, an enzyme anchored

in endothelial surfaces of the vascular system, leaving chemical structures at the end of carbohydrate conjugates which make them recognizable by asialoglycoprotein-receptors for endocytosis (Schauer, 1978 and 1987).

For some time sialic acids (superfamily of neuraminic acid derivatives) are gaining interest by being related to pathological processes. Enhanced serum concentrations of NANA were observed in connection with the incidence of tumors (Bellmann *et al.*, 1990; Fischer *et al.*, 1990) and inflammation (Yamamoto *et al.*, 1995) as well as bacterial and viral infections (Chappey *et al.*, 1995). Furthermore higher serum concentrations of NANA correlate positively with age while dramatic decreases of sialyltransferase activities and gangliosidic bound sialic acid residues in brain take place. There also appear to exist correlations between increased serum sialic acids and coronary artery diseases of diabetics (Yokoyama *et al.*, 1995; Pickup *et al.*, 1995) and atherosclerotic patients (Gracheva *et al.*, 1994).

In attempts to estimate the atherogenic risk of cholesterol enriched lipoproteins more and more attention is paid to the density of LDL particles and their biochemical properties. Positive correlations between the oxidizability of LDL and forms of higher density as well as a predominance of smaller LDL molecules in patients with atherosclerotic blood vessels have been described (Slyper, 1994; Dejager *et al.*, 1993). Roheim and Asztalos (1995) reported an increased oxidizability of smaller, dense LDL subfractions, while the smaller LDL molecules were found to contain

Abbreviations: LDL, low density lipoprotein; N-LDL, desialylated LDL; apoB-100, apolipoprotein B-100; NANA, N-acetyl-neuraminic-acid; Cu, copper; trp, tryptophan.

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smaller amounts of sialic acids (La Belle and Krauss, 1990; Tertov *et al.*, 1993). Hints for an increased atherogenicity of these particles are also given in an article of Taniguchi *et al.* (1989). They describe that neuraminidase-treated LDL is more actively metabolized by mouse macrophages than is untreated LDL. In earlier papers Orekhov *et al.* (1989) and Filipovic *et al.* (1979) showed that there is a strong negative correlation between the sialic acid content of LDL and its ability to promote intracellular lipid accumulation.

In this communication we present evidence that there are particular effects of desialylation, i.e. the (enzymatic) removal of sialic acid residues from the carbohydrate chains of the apoB-100 moiety, on oxidizability of LDL depending on the distinct compartment that is observed.

Materials and Methods

Chemicals and Biochemicals

Chemicals were obtained from SIGMA Chemical Co. (Munich) in the greatest available purity. Likewise, the enzyme neuraminidase EC 3.2.1.18 from *Clostridium perfringens* N-2876 was purchased from SIGMA (Munich).

LDL-preparation

Blood was drawn from two healthy, normolipemic male volunteers by venipuncture and EDTA was added (1 mg/ml final conc.). Plasma was prepared immediately by a low spin centrifugation and provided with sucrose (0.6%). (The two preparations were not pooled).

LDL was separated by a single-step ultracentrifugation (swinging bucket rotor, 120000×g, 24 h, 10 °C): 3 to 4 ml plasma were adjusted to a density of 1.41 g/ml with KBr and overlaid with density solutions of 1.080, 1.050 and 1.000 g/ml (KBr and 1 g EDTA/l each). LDL was stored (under nitrogen, 4 °C, in the dark) no longer than two weeks before use. Desalting into phosphate-buffered saline (160 mM NaCl, 10 mM sodium phosphate buffer pH 7.4) was performed by EconoPac DG-10 gel filtration columns.

LDL concentration (μM) was estimated on the basis of the solution's protein content (determined by means of the Coomassie Brilliant Blue microassay of BioRad) assuming that one LDL molecule

contains one molecule apoB-100 with a molecular weight of 514 kD. The α -tocopherol content of LDL preparations was determined by organic extraction and HPLC analysis (Nucleosil 300, methanol/water 95/5, fluorescence detection 292/335 nm) according to Esterbauer *et al.* (1992).

Desialylation

LDL preparations with diminished NANA-content were prepared by enzymatic hydrolysis of NANA. LDL was incubated (30 min, 37 °C, pH 5.5) with different amounts of neuraminidase (0– 2×10^8 U/mol apoB-100). The reaction was stopped by cooling in iced water and addition of KBr for LDL reisolation by ultracentrifugation.

Unit definition: 1 International Unit (U) is the amount of enzyme which converts 1 μmol of substrate per minute.

Determination of NANA

For determination of the NANA-content of LDL first the NANA residues had to be released from their linkage to the carbohydrate moiety of apoB-100: 25 μg LDL-protein (0.05 nmol) were incubated with 10 mU neuraminidase (30 min, 37 °C, pH 5.5). Separation from LDL and the enzyme was performed by ultrafiltration in microcon devices (Microcon-100 from Amicon).

From the filtrate NANA was determined spectrofluorometrically according to the procedure of Hammond and Papermaster (1976) in a slightly modified manner: 200 μl of the NANA containing solution were incubated (30 min, 37 °C) with 20 μl 1 N H₂SO₄ and 50 μl 0.025 M periodic acid in 0.125 M HCl. Thereafter 40 μl Na-arsenite (2% w/w in 0.5 N HCl) were added to reduce the remaining periodic acid. By 7.5 min boiling with 400 μl 0.1 M thiobarbituric acid (pH 9.0) the fluorophore was formed. It was extracted with 600 μl acidified n-butanol (5% w/w HCl) and the measurement of the fluorescence was performed in an Hitachi fluorescence spectrophotometer model F-4500 at an excitation wavelength of 550 nm and an emission wavelength of 570 nm.

Oxidation of LDL

EDTA-free LDL and desialylated LDL (N⁻LDL) respectively from different human donors were subjected to oxidation by Cu(II)-ions.

Formation of conjugated dienes was measured spectrophotometrically by monitoring the increase in absorbance at 234 nm immediately after addition of the CuSO_4 solution to the LDL solution (thermostatted cuvette holder, 37 °C). The final concentrations were 0.05 μM LDL/N⁻LDL and 1.67 μM Cu(II).

Electrophoretic mobility after incubation of LDL/N⁻LDL (0.73 μM) with Cu(II) (0.5 or 5.0 μM) for 24 h was determined by electrophoresis on 0.8% agarose gel. Before the run lipoprotein staining was performed by incubation of the reaction mixture (30 min, room temperature) with sudan black (200 mg in 5 ml ethanol, 3 ml glycerol 87%, 2 ml aq. bidest.).

Fluorescence quenching

LDL- and N⁻LDL-trp (tryptophan) fluorescence (lipoprotein concentration 0.1 μM) depending on increasing Cu(II) concentrations (0–13 μM) was measured according to Gießauf *et al.* (1995).

Results

Oxidation reactions induced by Cu(II) ions modify both compartments of the LDL particle. The core lipids undergo peroxidation which intermittently yields conjugated dienes and a number of aldehydic and other low molecular weight end products, while the apolipoprotein is

modified as a result of reactions of aldehydes with the ϵ -amino groups of lysyl amino acid residues (Brown und Goldstein, 1985). We tested different LDL preparations from two donors as to their optical (dieneconjugation, Fig. 1) and chromatographic (gel electrophoresis, Table I) behaviours after oxidative treatment with Cu(II) ions.

1) Prolongation of the lag phase of desialylated LDL in Cu(II)-mediated production of conjugated dienes

In the presence of 1.67 μM Cu(II) ions, the absorbance at 234 nm of three LDL preparations (0.05 μM) was monitored. The NANA content was 16.1, 13.7 and 6.2 mol/mol apoB-100 respectively; the α -tocopherol concentration was essentially the same in the three preparations (6 mol/mol LDL).

As shown in Fig. 1 the lag phase of the production of conjugated dienes measured as increase in absorbance was prolonged with diminished NANA content of the LDL. Besides no change in the propagation rate but a slightly diminished maximum diene production in the desialylated preparations could be observed.

2) Enhanced electrophoretic mobility of different LDL preparations after oxidative treatment with Cu(II) ions

The increase in the electrophoretic mobility of LDL on agarose gels reflects the sensitivity of the protein moiety towards oxidation triggered by copper ions. A modification of the positively charged ϵ -amino groups of the apolipoprotein exerts a more negative net charge of the particle which can be “quantified” by determination of the relative mobility on agarose gel. As demonstrated in Table I the oxidizability of LDL (0.73 μM) is enhanced by desialylation.

Depending on the blood donor and the copper concentration the effects differ in their extent. With respect to its antioxidative capacity it should be noted that LDL from donor B was deficient in vitamin E (3 mol/mol LDL) compared to that of donor A (6 mol/mol LDL, both preparations).

To get an idea of the existence and accessibility of copper binding sites at the apoB-100 there is a simple method provided by Gießauf *et al.* (1995). It is based on the fluorescence of tryptophan residues (trp) of the protein. Trp typically can be ex-

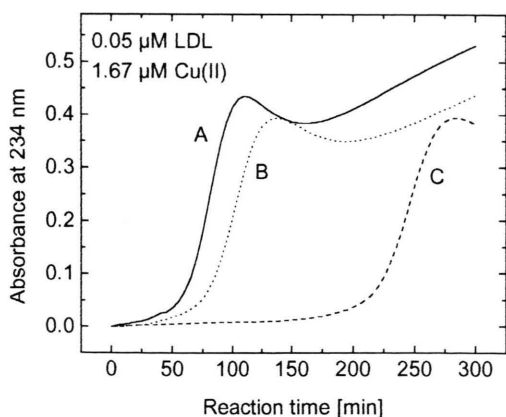


Fig. 1. Monitoring of dieneconjugation in low density lipoprotein caused by incubation with Cu(II) ions by continuous recording of changes in absorbance at 234 nm. A, untreated LDL (16.1 mol NANA/mol apoB-100). B, Partially desialylated LDL (13.7 mol NANA/mol apoB-100). C, 6.2 mol NANA/mol apoB-100.

Table I. Electrophoretic mobility of LDL 0.73 μM after 24 h incubation with Cu(II).

Donor	NANA content [mol NANA/mol apoB-100] (% of untreated LDL)		Cu(II) [μM]	Electrophoretic mobility [%] (0 μM Cu(II) = 100%)		Change in Cu(II)-enhanced electrophoretic mobility due to desialylation
	LDL	N ⁻ LDL		LDL	N ⁻ LDL	[%]
A	17	7 (38)	5.0	181.8+/-8.1	185.2+/-16.0	+ 3.4+/-17.9
A	17	7 (38)	0.5	149.3+/-8.7	175.4+/- 4.4	+26.2+/- 9.7
A*	18	5 (31)	0.5	166.7+/-3.7	196.1+/- 5.9	+29.4+/- 7.0
B	14	5 (36)	0.5	161.3+/-7.4	217.4+/- 4.3	+56.1+/- 8.6

*LDL from blood drawn after a fasting period.

cited by light of the wavelength 282 nm and has an emission maximum at 331 nm. 8 to 9 of the trp residues of apoB-100 seem to be oriented into the hydrophilic environment. Their fluorescence (21% of the total trp fluorescence of the LDL particle) can be quenched by copper ions if they bind next to the trp residues to the apolipoprotein.

3) Fluorescence quenching – Unchanged accessibility of certain binding sites for Cu(II) ions in LDL and desialylated LDL

We used this quenching technique to investigate whether copper binding is involved in the observations described above (1, 2). Two different LDL preparations were used and their fluorescence depending on increasing amounts of Cu(II) ions in the reaction solution was measured. As shown in Fig. 2 there exists no difference between untreated

(16.4 mol NANA/mol apoB-100) and desialylated (3.8 mol NANA/mol apoB-100) LDL (0.1 μM) in respect of quenching the trp fluorescence. The value can be reduced to about 80% indicating that all the trp residues in hydrophilic environment are accessible to the transition metal ions.

Discussion

Atherogenic processes seem to be initiated or augmented by oxidative modification of LDL (Schäfer-Elinder and Waldius, 1994; Ross, 1993) where structural configuration as well as the chemical composition of the macromolecule determines the oxidation resistance. Removal of sialic acid residues from LDL may influence charges and orientation of surface structures of the particle. Epidemiological findings and *in vivo* as well as *in vitro* studies provide growing evidence that there is a link between desialylation and atherogenicity of LDL. Dejager *et al.* (1993) showed that especially in dense LDL fractions the oxidation resistance seems to be diminished. Moreover Tertow *et al.* (1993) reported LDL species with low sialic acid contents exhibiting greater oxidizability and/or accumulating certain atherogenic lipids such as cholesterol. However understanding of the underlying mechanisms is still poor.

In our experiments using gel electrophoresis for analysis of LDL modifications by Cu(II) ions we could confirm the greater oxidizability of desialylated LDL compared to untreated LDL. In contrast we found an enhanced resistance towards production of conjugated dienes indicating peroxidizing unsaturated fatty acids in LDL. As changes in the electrophoretic mobility of LDL may repre-

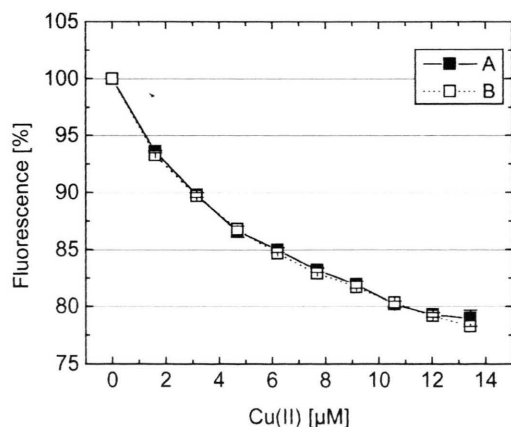


Fig. 2. Fluorescence quenching by increasing amounts of Cu(II) ions. A, untreated LDL (16.4 mol NANA/mol LDL); B, desialylated LDL (3.8 mol NANA/mol LDL). 100% = Total fluorescence of LDL due to tryptophan residues.

sent a modification of the apoB-100 moiety these findings are not necessarily contradictory. In agreement to our findings Hunt *et al.* (1994) reported that during the oxidation of probucol-enriched LDL modification of the apolipoprotein may occur without detectable lipid peroxidation. And Frey-Fressart *et al.* (1995) observed a reduced uptake of OH⁻-treated LDL via the apoB/E receptor of fibroblasts, although no lipidperoxidation and no enhancement of electrophoretic mobility took place. Considering the fact that desialylation

of glycoproteins is an essential step in their metabolic pathway determining the circulation time in the blood stream, simultaneously enhancement of apoB-100 modification that might increase its atherogenicity represents an intriguing situation.

As copper binding to the apolipoprotein, an initial event in LDL oxidation, does not seem to be affected by desialylation possibly the oxidation process is just "shifted" towards the protein moiety, however, the physiological consequences remain unclear.

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