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LDL resistance to oxidation: Effects of lipid phenotype, autologous HDL and alanine

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Abstract

Background: Although LDL resistance to copper-induced oxidation is a time-honoured method, how it is modulated by the physiologic variability of lipid phenotype and what influences the protective action of homologous HDL and exogenous alanine is still unclear.
Methods: In 159 subjects without severe dyslipidemias, LDL resistance to copper-induced oxidation (lag phase) was measured under standardised conditions, with alanine and with autologous HDL.
Results: Lag phase was normally distributed and averaged 68±10 min (range: 40–105 min). Both VLDL-triglycerides (37±5, 52±7, 59±7, 53±5 mg/dl, $p<0.05$) and LDL-triglycerides (27±2, 27±1, 30±2, 35±3 mg/dl, $p<0.01$) increased across quartiles of lag phase. The relative LDL enrichment in triglycerides (triglycerides percent or triglycerides/cholesterol ratio) was strongly related to lag phase ($r=0.29$ and $r=0.31$, $p<0.0005$ for both) independently of age, gender, BMI, and presence of diabetes or hypertension. The protective effect of HDL was variable (+42±18 min) and largely dependent on the capacity of HDL to resist oxidation ($r=0.69$, $p<0.0001$). Alanine induced a rather constant lag phase prolongation (+32±7 min) that was weakly related only to baseline lag phase ($r=0.17$, $p<0.05$).
Conclusions: Relative triglyceride abundance protects LDL from *ex-vivo* oxidation, HDL particles protect LDL mainly through substrate dilution and alanine probably through a direct anti-oxidant effect.

Keywords: LDL oxidation; Lag phase; HDL oxidation; Anti-oxidants

1. Introduction

The chemical modification of low-density lipoproteins (LDL) induced by oxidative reactions is a crucial step in the atherosclerotic process [1–3]. During its lifespan, and especially in their traffic within the arterial wall, LDL particles are exposed to a wide range of chemical insults, the net effect of which depends on the balance between the intensity of the challenge and the efficiency of the defence mechanisms. When oxygen radicals are overproduced – and/or lipoprotein particles are more vulnerable to this injury – the oxidative process induces modifications of the Apo-B protein that make the LDL particle less efficiently handled by the physiologic B-E receptor-mediated pathway and more prone to accumulate into macrophages through the scavenger pathway [4]. LDL particles are physiologically protected from oxidation by the presence of anti-oxidants in the aqueous ambient or within their lipid layer. The vulnerability to oxidative modification has traditionally been estimated *ex vivo* by measuring the generation of conjugated dienes upon challenging LDL particles with a strong pro-oxidant such as copper or iron [5]. By using this technique, studies have confirmed the relevance of anti-oxidants in LDL susceptibility to oxidative damage [6]. Despite the relative constancy of vitamin intake and also of LDL vitamin content in homogeneous populations, LDL particles still show a wide range of susceptibility degrees to oxidative stress [7]. The fatty acids composition of triglycerides (TG, monounsaturated *vs* polyunsaturated or saturated) [8] and the particle size/density (small dense *vs* large buoyant) [9] have been proposed as factors responsible for the variable LDL resistance to oxidation. However, in quantitative terms the impact of either aspect

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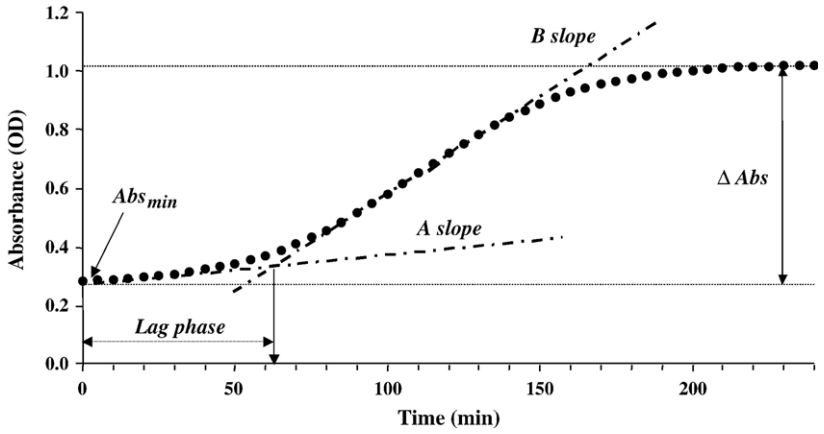

Fig. 1. Scheme of the kinetics of conjugated dienes formation from LDL particles incubated with copper. Abs_{min} indicates the absorbance at time 0, A slope and B slope indicate the slope of the first and second linear portion of the curve, respectively, ΔAbs indicates the absolute change in the absorbance relative to time 0.

results modest and a large proportion of the biological variability remains unexplained.

In vivo clearly several factors modulate LDL oxidability and among these HDL appears to play a relevant role showing a strong protective effect as documented by the prolongation of the lag phase in experiments in which the two substrates were simultaneously exposed to oxidation[10–13]. Although this effect has been traditionally ascribed to the presence of anti-oxidant enzymes (such as paraoxonase or platelet-activating factor acetylhydrolase), no study has directly verified this hypothesis nor other mechanisms have been searched.

Finally, rather than LDL susceptibility to oxidation the degree of spontaneously occurring LDL oxidation/modification would probably better reflect the extent of *in-vivo* oxidative stress. Unfortunately, the direct determination of these minimally modified LDL particles is hampered by technical difficulties. The protective effect of alanine on LDL oxidisability has been proposed as a valid alternative to estimate the amount of lipid peroxides present in native LDL particles [14]. However, whether this specific characteristic of alanine, which has been inferred from its chemical properties (its antioxidant action being less effective when lipid peroxides are abundant) also depends on lipoprotein composition has not been investigated.

This study was set forth to establish whether LDL susceptibility to oxidation is influenced by differences in lipid and/or protein composition and to describe what affects the protective effect of either HDL and alanine.

2. Methods

2.1. Study subjects

Plasma samples were taken after an overnight (10–14 h) fast from a total of 159 consecutive patients attending our outpatient clinic in whom a complete clinical work-up had excluded significant liver, kidney and thyroid dysfunction, severe hypertriglyceridaemia (serum TG >250 mg/dl) or hypercholesterolaemia (serum total cholesterol >250 mg/dl), systemic inflammatory diseases or current treatment with anti-oxidants, statins, and fibrates. Patients with impaired fasting glucose (≥5.6 mM and <7 mM) underwent a standard 75 g oral glucose tolerance test to establish the diagnosis.

2.2. Measurement of LDL and HDL oxidation

For the measurement of *in-vitro* LDL and HDL oxidation, we followed the experimental protocol described by Esterbauer et al. [5] with some modifications. Briefly, blood (10 ml) was collected in tubes containing EDTA (1 mg/ml) and immediately centrifuged at 3000 rpm at 4 °C. Very-low density and intermediate-density (VLDL-IDL), low-density (LDL), and high-density (HDL) lipoproteins (d=1.006–1.019, 1.019–1.063 and 1.063–1.210, respectively) were isolated by sequential ultracentrifugation at 42,000 rpm at 4 °C (OPTIMA L-90 K, Beckman, CA, USA) in NaBr solutions with EDTA to avoid oxidation during isolation. All solutions were degassed and samples were kept at 4 °C in a dark environment. To remove EDTA, lipoprotein fractions were gel-filtered on a Sephadex column (Econo-Pac 10 DG columns; Bio-Rad), and eluted in 1M PBS buffer, pH 7.4. LDL particle concentration was adjusted at 50 μg of protein per ml by measuring total protein with the bicinchoninic acid method before a freshly prepared aqueous copper solution ($CuSO_4$, final concentration 1.0 μM) was added. The kinetics of conjugated dienes formation were followed spectrophotometrically at 234 nm at 37 °C every 1.5 min for 4 h and expressed as optical density (OD) units. A typical copper-induced LDL oxidation curve shows three consecutive phases: the lag phase, the propagation phase and the decomposition phase. The lag phase is defined as the time at which the first phase (A) and second phase (B) cross each other (Fig. 1). Once the experimental conditions are maintained constant, this time length depends on the intrinsic properties of the LDL particle (*e.g.*, antioxidant content, size, pre-formed lipid peroxides). The *y*-axis intercept of the A slope was defined as minimal absorbance (Abs_{min}) and the maximal increment above this value reached during the experiment was defined as ΔAbs.

The antioxidant effect of alanine on *in-vitro* LDL oxidation was tested in separate experiments by measuring the lag phase in the presence of 0.05 mM alanine. For the measurement of HDL lag phase we followed the same protocol as for LDL but the HDL protein concentration was set at 68 μg/ml. The effect of HDL on LDL oxidation was measured as the lag phase of LDL oxidation in a

Table 1
Characteristics of the study subjects

	Mean±SD	Range
n	159	
Gender (M/F)	83/76	
Age (years)	47±12	24–71
BMI (kg/m^2)	26.4±4.6	18.0–41.0
Total serum cholesterol (mg/dl)	187±36	82–250
Serum LDL-cholesterol (mg/dl)	117±32	31–189
Serum HDL-cholesterol (mg/dl)	52±16	20–96
Serum triglycerides (mg/dl)	90±46	34–250