Rapid communication

# Dialysis of isolated low density lipoprotein induces a loss of lipophilic antioxidants and increases the susceptibility to oxidation in vitro

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## Abstract

We determined the effects of different dialysis conditions on the antioxidant content, duration of the lag phase and oxidation rate of LDL. Dialysis for 22 h resulted in a 56%–66% reduction in the concentrations of $\beta$-carotene, lycopene and $\alpha$-tocopherol. The lag phase of copper-induced oxidation of freshly isolated LDL was considerably longer than that of LDL dialysed for 22 or 44 h. Our data show that dialysis may result in LDL preparations with antioxidant compositions that are not truly representative of freshly isolated lipoproteins.

## 1. Introduction

Several lines of evidence, from both in vitro and in vivo studies, suggest that oxidative modification of low density lipoprotein (LDL) may contribute to atherosclerosis [1–4]. A number of assays monitoring the oxidative susceptibility of the particle have been developed to evaluate the atherogenic potential of LDL [5–7]. Most often the LDL oxidation is initiated by incubating with cultured cells or with transition metal ions as pro-oxidants [5]. Esterbauer et al. [8] developed a method to assess the susceptibility of LDL to copper-mediated oxidation in vitro by continuous monitoring of the formation of conjugated dienes. The mechanistic aspects of this method were investigated recently [9,10]. The in vitro assay of conjugated diene formation is widely used to estimate the resistance of LDL to oxidation (e.g. [12–14]). This implies that the isolated LDL

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should be representative of the circulating LDL particles in vivo.

Unfortunately, ultracentrifugation, the most widely used procedure for LDL isolation, is time-consuming and preservatives like EDTA are regularly included in the high-salt solutions to limit oxidation. Isolated LDL are often extensively dialysed in order to remove these compounds prior to the oxidation assay. In this study we examined the effects of this dialysis step on the concentration of lipophilic antioxidants in LDL and on the parameters of the diene formation during oxidation of LDL in the presence of $Cu^{2+}$.

## 2. Methods

### 2.1. Subjects

Nine healthy volunteers (6 males, 3 females) participated in the study after informed consent. Ages ranged from 25 to 50 years. The average plasma total cholesterol level was 5.4 mmol/l (range 4.2–5.3 in females and 4.4–6.6 in males). These and other characteristics of the study subjects are given in Table 1.

### 2.2. Lipoprotein isolation, preparation and oxidation

Blood samples were obtained, after an overnight fast, in EDTA-containing tubes. Plasma was prepared by centrifugation at 2000 × $g$ for 15 min at 4°C. The plasma samples were divided into two identical aliquots and lipoproteins were separated by density gradient ultracentrifugation [15], using a Beckman SW40 or SW41 rotor and ultraclear centrifuge tubes. After ultracentrifugation, the LDL fractions ($d$ = 1.019–1.063 g/ml) were collected by tube slicing. A part of the freshly isolated LDL in 0.1 mM EDTA was immediately used for a conjugated diene formation assay [14]. Another part was stored immediately at − 80°C under nitrogen until analysis of antioxidants. LDL isolated in 1.0 mM EDTA were dialysed against 0.01 mM EDTA according to Kleinveld et al. [10].

Three different dialysis methods were used, varying in time and/or EDTA concentration. In all dialysis procedures 2 ml LDL samples were dialysed at 4°C in the dark against a total volume of 3 l of 0.01 M phosphate-buffered saline (PBS,

pH 7.4). All dialysis solutions were degassed and purged with nitrogen prior to use and during dialysis. In method A, LDL was dialysed for 22 h against PBS containing 0.01 mM EDTA. In methods B and C, LDL were dialysed for 44 h against PBS with 0.01 mM EDTA (method B) or without EDTA (method C). Immediately after dialysis the protein content of LDL was determined using bovine albumin as the standard [16], and the LDL samples were stored at − 80°C, under nitrogen, until antioxidant measurement.

The formation of conjugated dienes during copper-mediated oxidation was continuously monitored at 234 nm in a Hitachi U 2000 UV/VIS spectrophotometer. Before the start of the oxidation experiments using dialysed LDL, samples were diluted with PBS to give a final concentration in the assay cuvette of 50 $\mu$g/ml LDL protein and 1 $\mu$M EDTA. The incubation mixture was brought to 30°C and $CuCl_2$ was added to a final concentration of 5 $\mu$M to initiate oxidation. The absorbance at 234 nm was monitored at 2 min intervals and the lag phase before conjugated diene formation occurred, as well as the maximum rate of oxidation and the maximal amount of conjugated dienes, was calculated from the oxidation profile [8]. When dialysis was omitted, LDL samples were diluted with 1.18 M NaCl containing 0.1 mM EDTA, immediately after lipoprotein isolation. This ensured that the EDTA concentration was equal in all samples. $CuCl_2$-mediated oxidation of undialysed LDL was carried out according to Princen et al. [14]. Briefly, 50 $\mu$g of LDL protein was incubated at 30°C in a total volume of 1.0 ml PBS containing 50 $\mu$M $CuCl_2$

Table 1
Characteristics of subjects

|  | Mean ± S.E.M. | Range |
| --- | --- | --- |
| Age (years) | 37.8 ± 2.7 | 25–50 |
| BMI (kg/m²) | 22.1 ± 0.8 | 18.9–25.0 |
| Plasma cholesterol (mM) | 5.42 ± 0.82 | 4.14–6.64 |
| Plasma TAG (mM) | 1.08 ± 0.28 | 0.71–1.50 |
| $\beta$-carotene (mg/l) | 0.33 ± 0.13 | 0.11–0.57 |
| Lycopene (mg/l) | 0.24 ± 0.12 | 0.09–0.52 |
| $\alpha$-tocopherol (mg/l) | 11.21 ± 2.23 | 8.34–14.80 |

Data represent the mean of 9 individuals. BMI, body mass index; TAG, triacylglycerol.

Table 2
Effect of dialysis on lipid-soluble antioxidants in LDL ($\mu$g per mg LDL protein)

|                | No dialysis            | 22 h + EDTA     | 44 h + EDTA     | 44 h − EDTA     |
|----------------|------------------------|-----------------|-----------------|-----------------|
| $\alpha$-tocopherol | $6.49 \pm 0.50$[a] | $2.19 \pm 0.31$ | $3.00 \pm 0.31$ | $2.46 \pm 0.31$ |
| $\beta$-carotene   | $0.36 \pm 0.04$[a] | $0.16 \pm 0.04$ | $0.19 \pm 0.02$ | $0.13 \pm 0.03$ |
| Lycopene           | $0.26 \pm 0.05$[a] | $0.09 \pm 0.02$ | $0.12 \pm 0.03$ | $0.04 \pm 0.01$ |

Data represent the mean $\pm$ S.E.M. ($n = 9$).
[a]Significantly different from dialysed LDL, $P < 0.001$.

and conjugated diene formation was monitored at 234 nm. The final EDTA concentration was 25 $\mu$M.

### 2.3. Analytical procedures

The concentrations of $\beta$-carotene, lycopene and $\alpha$-tocopherol were determined in plasma and LDL as follows. LDL or plasma samples were extracted with n-heptane containing $\delta$-tocopherol and ethyl-$\beta$-apo-8'-carotenoate as internal standards. Lipid soluble antioxidants were determined by HPLC using an ET 200/8/4 nucleosil 5CN column (250 × 4 mm, Mackery and Nagel) and n-heptane–chloroform–2-propanol (100:1.5:0.15, v/v) as the mobile phase. The eluent was monitored with a UV/VIS detector set at 292 nm ($\alpha$-tocopherol), 298 nm ($\delta$-tocopherol) and 450 nm (carotenoids).

Total cholesterol and triacylglycerol in plasma and lipoprotein fractions were measured by standard enzymatic procedures (Boehringer, Mannheim, Germany) on a Cobas Mira S analyzer. Malondialdehyde (MDA) in LDL was determined by HPLC analysis after complexation with diethyl barbituric acid essentially as described by Wong et al. [17]. Lipoperoxides in LDL were measured with a iodometric assay [18].

### 2.4. Statistical analysis

Results are expressed as the mean $\pm$ S.E.M. ($n = 9$). Statistical analysis was performed with the SAS statistical package. Data were analysed by one way ANOVA. Multiple comparisons between means were carried out using the Tukey test.

## 3. Results and discussion

In the present study we examined the effect of dialysis on the antioxidant content of LDL and on the susceptibility of LDL to copper-initiated oxidation. The data shown in Table 2 clearly indicate that dialysis of LDL resulted in a significant loss of $\alpha$-tocopherol, the major lipophilic antioxidant in LDL. After 22 h of dialysis, in the presence of 0.01 mM EDTA, the concentration of $\alpha$-tocopherol was decreased by approximately 66%. Prolonged dialysis for up to 44 h, in either the presence or absence of EDTA, did not further decrease LDL $\alpha$-tocopherol levels. Analysis of $\alpha$-tocopherol in all lipoprotein fractions gave a recovery of approximately 100% (not shown). The loss of $\beta$-carotene and lycopene from the LDL particle was comparable to that of $\alpha$-tocopherol (Table 2).

It was reported previously that, in LDL subjected to copper-mediated oxidation, depletion of lipophilic antioxidants precedes the propagation phase of the oxidation of polyunsaturated fatty acids [9]. This implies that the dialysis-induced loss of fat-soluble antioxidants may significantly affect the lag phase of oxidised LDL. To allow direct measurement of the oxidation of freshly isolated LDL in the presence of EDTA (25 $\mu$M), we determined the optimal copper concentration in the assay mixture. The optimum was found to be 50 $\mu$M, close to the 40 $\mu$M reported by Princen et al. [14].

The optimal $CuCl_2$ concentration for the oxidation of dialysed LDL particles in the presence of 1 $\mu$M EDTA at protein concentration of 50 $\mu$g/ml was determined to be 5 $\mu$M. This agrees with published data [10], although there is ongoing

discussion on the precise optimal molar ratio of $Cu^{2+}/LDL$ [11]. The results presented in Fig. 1 demonstrate that dialysis of LDL for 22 or 44 h in the presence of 0.01 mM EDTA significantly decreased the lag phase ($P < 0.001$) compared with undialysed LDL. Omission of EDTA in the dialysis buffer did not result in a further decrease of the lag time observed after 44 h of dialysis. Neither the maximal rate of oxidation (Fig. 1) nor the maximal amount of conjugated dienes formed during oxidation was profoundly changed after dialysis. The maximal rate of oxidation was increased by about 25% after 44 h dialysis in the presence of EDTA. The reason for this increase is not clear at present.

As an estimation of the lipid peroxidation of polyunsaturated fatty acids during dialysis, we measured the level of MDA in LDL before and after dialysis. Freshly isolated LDL contained $1.04 \pm 0.35$ nmol MDA per mg protein. This value was virtually unchanged after 22 h of dialysis ($1.34 \pm 0.17$ nmol/mg). After dialysis for 44 h in the presence of EDTA the level of MDA tended to increase ($2.02 \pm 0.29$), while after 44 h in the absence of EDTA, MDA levels in LDL were significantly increased compared with the other dialysis conditions ($3.19 \pm 0.28$ nmol/mg protein, $P < 0.001$). The lipoperoxide levels in the same LDL preparations were $42 \pm 2$, $82 \pm 5$, $95 \pm 3$ and $372 \pm 68$ nmol per mg LDL protein, respectively. Again the latter values are significantly increased compared with the other conditions ($P < 0.001$). Fig. 2 illustrates our findings on MDA and lipoperoxide contents of freshly isolated and dialysed LDL.

It must be kept in mind that not all lipophilic antioxidants present in plasma are exclusively present in LDL. Freshly isolated VLDL, LDL and HDL of our study subjects contained $13 \pm 6\%$, $45 \pm 5\%$ and $30 \pm 7\%$ of total plasma $\alpha$-tocopherol, respectively. The remaining $12 \pm 2\%$ was located in the lipoprotein deficient plasma fraction of density $> 1.21$ g/ml. So both
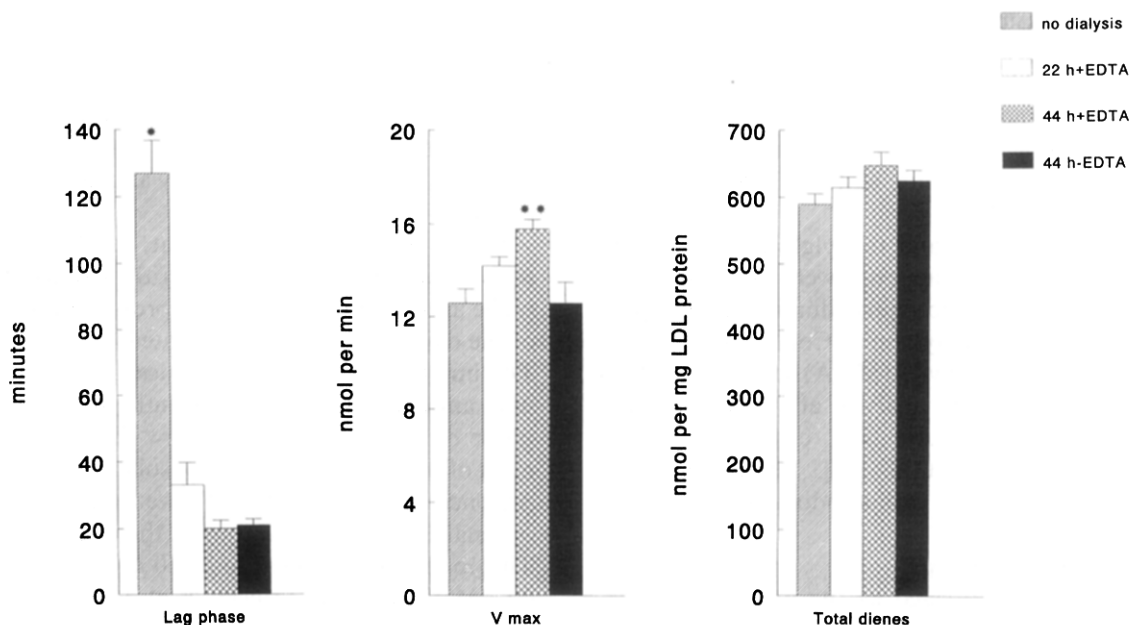


Fig. 1. Bar diagram showing lag phase before conjugated diene formation, the maximum oxidation rate and the maximal amount of dienes formed in copper-oxidised LDL which were not dialysed; dialysed for 22 h in the presence of EDTA (method A); dialysed for 44 h in the presence of EDTA (method B) or dialysed for 44 h in the absence of EDTA (method C). Values represent the mean $\pm$ S.E.M. of LDL samples from 9 subjects. *No dialysis significantly different from all dialysis groups, $P < 0.001$. **Significantly different from no dialysis, $P < 0.01$.
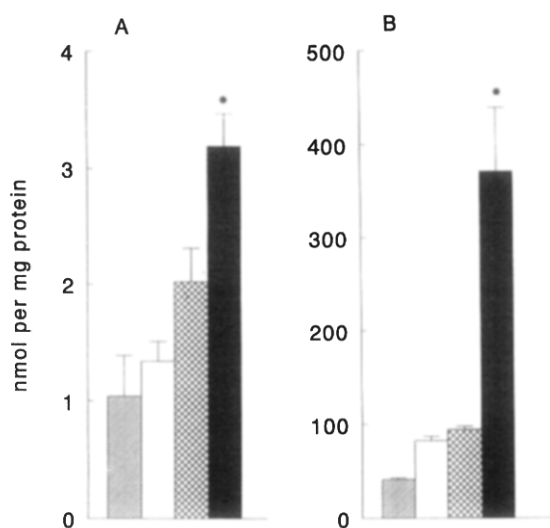
Fig. 2. The effect of different dialysis conditions on the concentration of malondialdehyde (A) and lipoperoxides (B) in LDL. LDL were not dialysed (hatched bars); dialysed for 22 h in the presence of EDTA (method A, open bars); dialysed for 44 h in the presence of EDTA (method B, double hatched bars) or dialysed for 44 h in the absence of EDTA (method C, closed bars). Values represent the mean ± S.E.M. of LDL samples from 9 subjects. *Significantly different from no dialysis, $P < 0.001$.

LDL and HDL are major carriers of $\alpha$-tocopherol in normolipidemic plasma. Expressed on a cholesterol basis LDL and HDL have 1.8 ± 0.3 and 3.3 ± 0.4 $\mu$g $\alpha$-tocopherol/$\mu$mol cholesterol, respectively. It is conceivable that $\alpha$-tocopherol or other antioxidants from HDL may help to prevent LDL oxidation under in vivo conditions.

In summary, our data clearly show that dialysis of LDL results in a depletion of lipophilic antioxidants and in a decrease in the lag phase of copper-initiated oxidation of LDL particles. Antioxidants such as ubiquinol-10, not measured in the present study, could also be affected by dialysis. It can be concluded that dialysis may result in LDL particles that are not truly representative of freshly isolated lipoproteins. Future research should be directed towards the development of highly controlled, rapid procedures for LDL isolation. Our data show that it may be prudent not to submit LDL to prolonged dialysis. Recently Puhl et al. [11] have proposed gelfiltration as the standard procedure for removal of salts from LDL samples.

## Acknowledgements

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