The effect of heparin on Cu^{2+}-mediated oxidation of human low-density lipoproteins

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Abstract The effect of heparin (HE) on the susceptibility of human low-density lipoprotein (LDL) to Cu^{2+}-induced oxidation was investigated by monitoring conjugated diene formation. HE did not modify the maximum formation of conjugated diene, but increased markedly the lag phase. The plot of change in oxidation rate vs. time showed that the absolute value of V_{max} was dependent on Cu^{2+} concentration and that HE increased the time necessary to reach V_{max}. The value of constant K (the Cu^{2+} concentration producing a t_{lag} of twice the minimum value) increased in the presence of HE, whereas the value of t_{min} (the time theoretically required for LDL oxidation at an infinite Cu^{2+} concentration) was not substantially affected. These results indicate that HE might play a protective antioxidant effect on LDL, probably affecting both the structural properties of the particle and the amount of Cu^{2+} available for the oxidation.

Key words: Heparin; Low-density lipoprotein; Copper oxidation; Kinetics

1. Introduction

The lipid deposition in arterial wall by low-density lipoprotein (LDL) is a critical point in the development of atherosclerotic degeneration. Oxidative modifications of LDL are likely to increase its atherogenic potential, since macrophages and smooth muscle cells in culture avidly accumulate oxidized LDL quite independently from intracellular cholesterol level, leading to foam cell formation [1,2]. Moreover, oxidized LDL may promote thrombosis, acting on both endothelial cells and platelets [2].

LDL oxidation probably occurs in the arterial wall, where it is excluded from the antioxidant activity of plasma [1,2]. Moreover, atherosclerotic arterial wall contains increased levels of copper and iron ions, which induce LDL oxidation [3]. In this compartment, LDL may interact also with proteoglycans (PGs), forming complexes that are taken up avidly by macrophages [4,5]. The interaction of LDL with hexuronate-containing sulphated glycosaminoglycans was recently shown to modify the structural properties of the particle, heparin (HE) being particularly effective [6].

On this basis, we studied whether the susceptibility of LDL to oxidation might also be affected by HE, which was found to decrease lipid-free radical peroxidation [7].

As experimental model, we used the highly reproducible LDL oxidation mediated by Cu^{2+} [8,9]. In this model, the lag time of LDL oxidation decreases with increasing Cu^{2+} level, according to a relationship described by an equation formally similar to that of Michaelis and Menten for enzyme kinetics [10].

2. Materials and methods

Human LDL was prepared from fresh pooled EDTA plasma of normolipemic donors by ultracentrifugation using a single-step discontinuous gradient in a Beckman SW 41 rotor at 38,000 rpm for 20 h at 10°C, according to Dieber et al. [11] with minor modifications. LDL was collected as the fraction floating at a density of 1.019 to 1.063 g/ml and used within 1 week from the isolation. Its mean composition was as follows: 26.0 ± 1.3% proteins, 15.8 ± 0.8% phospholipids, 48.7 ± 2.4% total cholesterol, 9.5 ± 0.5% triglycerides.

Protein concentration was determined according to Smith et al. [12]. Total cholesterol, phospholipid and triglyceride contents were assayed by standard enzymatic methods (Boehringer Mannheim).

The mean diameter of LDL resulted of 20 nm, as determined by quasi elastic laser light scattering [13] using a BI 90 particle sizer (Brookhaven Instrument Corporation, Holstville, NY), equipped with a laser source at an excitation wavelength of 632.8 nm.

Before oxidation, LDL was desalted by exhaustive dialysis against 10 mM phosphate-buffered saline (PBS), pH 7.4, at 4°C in the dark. Samples of LDL corresponding to 80 μg cholesterol/ml were then oxidized with 0.9, 1.8 and 3.6 μM $CuSO_4$. These concentrations are in the range originally used by Gieseg and Esterbauer [10] and in the same order of magnitude as those described in atherosclerotic plaques [3]. The oxidation was performed in PBS at 30°C, in the absence and in the presence of HE (varying from 150 to 750 μg/ml, corresponding to 72–362 μg/ml of hexuronate) (Sigma). The oxidation was monitored continuously by measuring the increase in absorbance at 234 nm due to the formation of conjugated CC double bonds [14].

The water used to make up reagents was purified from inorganic and organic contaminants by an Elga-Stat UHQ apparatus (Wycombe Bucks, UK). All other reagents were of analytical grade.

3. Results

The kinetics of Cu^{2+}-induced oxidation of human LDL were followed by determining the curves of diene vs. time profile. In the absence of HE, the decrease of Cu^{2+} concentration in the reaction mixture was coupled with an increase of the value of lag time (t_{lag}), determined graphically by the intercept of the tangents to the slow and fast increase of the diene absorption [10] (Fig. 1, open circles). In the absence of HE, the values of t_{lag} were as follows: 61 min at 3.6 μM $CuSO_4$; 84 min at 1.8 μM $CuSO_4$ and 110 min at 0.9 μM $CuSO_4$. The presence of HE in the incubation mixture (Fig. 1, black circles) did not modify the maximum formation of conjugated dienes, but increased significantly t_{lag}. This increase was progressively higher at lower Cu^{2+}

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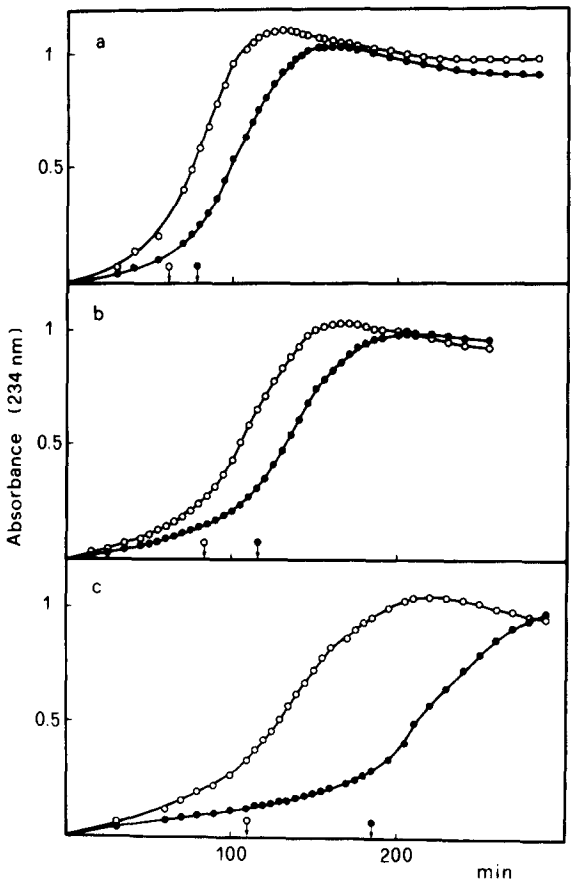

Fig. 1. Effect of heparin on the kinetic of Cu^{2+}-induced oxidation of human LDL. Human LDL (80 μg/ml as total cholesterol) in 10 mM PBS was incubated with different $CuSO_4$ concentrations (3.6 μM: a; 1.8 μM: b; 0.9 μM: c) in the absence (○-○) and in the presence (●-●) of 150 μg/ml HE. The formation of u.v.-absorbing conjugated dienes was followed recording the increase in 234 nm absorbance in 1-cm cuvette at 30°C. The initial absorbance was subtracted from all data. The value of t_{lag} (in the absence of HE: ↕; in the presence of 150 μg/ml HE: ↕) was determined graphically as reported in section 2. Similar results were obtained in three independent experiments, using different LDL preparations.

concentrations. Preliminary experiments showed that HE was effective above the concentration of 150 μg/ml. In the presence of 150 μg/ml HE, t_{lag} values were: 78, 116, 185 min at 3.6, 1.8, 0.9 μM Cu^{2+}, respectively. When higher amounts of HE were employed, the value of t_{lag} increased very markedly and in the presence of the lowest Cu^{2+} concentrations the propagation phase of lipid peroxidation was even incomplete after 350 min incubation, preventing the determination of t_{lag} (data not shown).

The first derivative of diene formation vs. time profiles was then calculated to obtain the change of oxidation rates as a function of time. The calculation was performed by the method of forward difference numerical derivation. These data are reported in Fig. 2, showing that in the absence of HE (open circles) the time required to reach the maximum velocity (V_{max}) of lipid peroxidation increased constantly with decreasing Cu^{2+} concentration (77, 103, 129 min at 3.6, 1.8, 0.9 μM $CuSO_4$, respectively).

The presence of 150 μg/ml HE in the incubation mixture (Fig. 2, black circles) delayed the reaching of V_{max} and this delay was higher at lower Cu^{2+} concentrations. Under these experimental conditions, V_{max} was reached after 89, 133, 205 min at 3.6, 1.8, 0.9 μM $CuSO_4$, respectively.

The decrease of Cu^{2+} concentration was coupled with a decrease of V_{max} value both in the absence and in the presence of HE. Moreover, the presence of HE decreased the value of V_{max} only when the highest Cu^{2+} concentration (3.6 μM) was employed (Fig. 2a). Kinetic parameters were further elaborated according to Gieseg and Esterbauer [10], who recently showed that the relationship between t_{lag} and Cu^{2+} concentration can be quantitatively described by the following equation which has the same functional form as the Michaelis and Menten equation:

$$\frac{1}{t_{lag}} = \frac{1}{t_{min}} \cdot \frac{Cu^{2+}}{K + [Cu^{2+}]}$$

The values of K (the copper concentration producing a t_{lag} of twice the minimum value) and t_{lag} minimum (t_{min}, the time theoretically required for LDL oxidation at an infinite Cu^{2+} concentration) were graphically determined from the double reciprocal plot of t_{lag} vs. $1/Cu^{2+}$. The value of K was given by the negative reciprocal of the x-axis intercept and the value of t_{min} by the y-axis intercept [10]. K value resulted 1.2 μM Cu^{2+} in the absence of HE and 2.5 μM Cu^{2+} in the presence of 150 μg/ml HE. On the contrary, the value of t_{min} was not substantially affected by the presence of HE, resulting always in 50 min.

4. Discussion

This study reports evidence that HE shows a significant protective effect on Cu^{2+}-induced oxidation of human LDL. The presence of HE in the reaction mixture increased both the lag phase and the time required to reach V_{max} of lipid oxidation. This effect was more marked with decreasing Cu^{2+} concentration.

The determination of t_{min} gave the same result both in the absence and in the presence of HE indicating that at an infinite Cu^{2+} concentration HE does not affect the length of lag phase. On this basis, it might be suggested that the effect of HE on the lag phase is founded on a competitive mechanism, since the protective antioxidant effect of HE is removed by the increase of Cu^{2+} concentration in the reaction mixture.

As recently shown by Gieseg and Esterbauer [10], the decrease of Cu^{2+} concentration was coupled with a decrease of V_{max} of conjugated diene formation, which is an index of the propagation phase and depends only on the lipid component of LDL. Under our experimental conditions, the addition of HE reduced the value of V_{max} only in the presence of the highest Cu^{2+} concentration. It was recently demonstrated that the interaction of HE with LDL induced a significant decrease of the microviscosity of LDL hydrophobic region, affecting also the thermotropic properties of both protein and lipid components [6]. These results indicate that the interaction of LDL with HE is likely to modify the structural properties of the particle. On this basis, we suggest that the structural modifications induced by HE on LDL might allow a propagation rate of Cu^{2+}-mediated oxidation which cannot reach the corresponding value of native LDL. This phenomenon is evident at the highest Cu^{2+} concentration, which corresponds to the highest value of V_{max}.

However, the protective effect of HE on LDL oxidation is

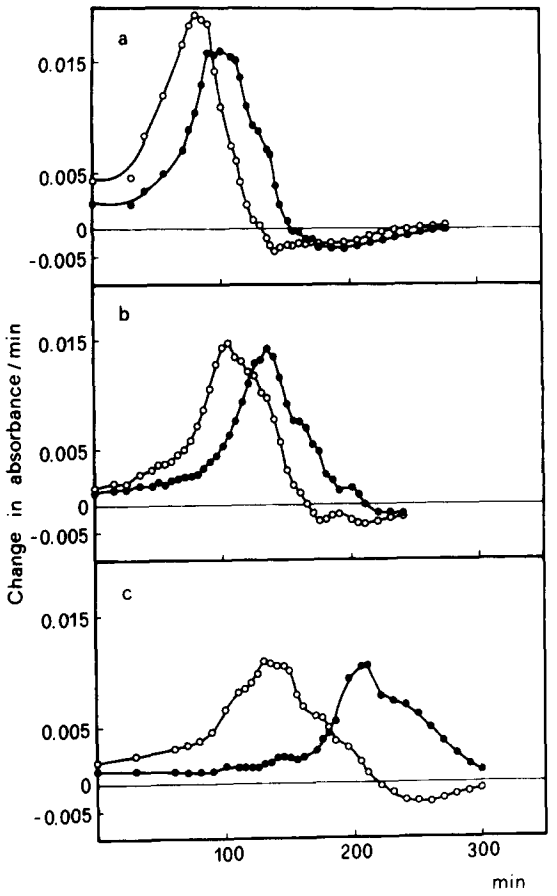

Fig. 2. Change of rates of Cu^{2+}-induced LDL oxidation as a function of time. The values were obtained determining the first derivative of diene formation vs. time profiles reported in Fig. 1. Control LDL, incubated in the presence of $CuSO_4$ only: ○-○; LDL incubated in the presence of both $CuSO_4$ and 150 μg/ml HE: ●-●. $CuSO_4$ concentration: 3.6 μM: a; 1.8 μM: b; 0.9 μM: c. The data were smoothed by a moving average calculation (average change in 234 nm absorption over 10 min) to reduce the noise in the plot.

not limited to the structural modifications of the particle, which probably involves a different accessibility of the lipid component to the oxidative process. HE is likely to affect also the amount of Cu^{2+} available for the oxidation, as suggested by the marked increase of the constant K in the presence of HE. The value of K indicates the Cu^{2+} concentration at which half of the pro-oxidative Cu^{2+}-binding sites are occupied [10]. Under our experimental conditions, LDL are employed at a concentration of 80 μg cholesterol/ml equal to 0.1 μM, according to Gieseg and Esterbauer [10]; therefore, the rate of initiation is half the maximum at 14 Cu^{2+} ions/LDL particle in the absence of HE. The addition of HE requires 25 Cu^{2+} ions/LDL to reach the same oxidative effect. This phenomenon could be explained on the basis of two different mechanisms: (1) a partial sequestration of Cu^{2+} by the polyanion chain of HE; (2) the possible change in the ability of LDL to bind Cu^{2+}, due to the structural modification of the particle following the interaction with HE.

HE was shown to inhibit Fe^{2+}-catalyzed peroxidation of γ-linolenic acid [7]. Our study emphasizes that the antioxidant effect of HE protects not only a free fatty acid, but also a biological structure, such as human LDL against Cu^{2+}-induced oxidation. This model of oxidation was demonstrated to produce LDL sharing many structural and functional properties with LDL oxidized by cells [15] or extracted from atherosclerotic plaques [16]. Moreover, it should be reminded that Cu^{2+} ions are contained in aortic wall and increase in atherosclerotic plaques [3], where they may contribute to the development of the lesion by oxidative stress. On this basis, we suggest that HE (and probably other glycosaminoglycans occurring in native PGs) might play a regulatory role in the process of LDL oxidation in vivo, which may change the metabolic fate of the particle, increasing its atherogenic potential [1,2].

HE is largely employed in anticlotting treatment even for relatively long periods of time. Our paper raises the question whether HE could also play an antithrombotic role, decreasing the formation of oxidized LDL which are known to exhibit a prothrombotic activity [1,2].

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