Heparin protection against Fe^{2+}-and Cu^{2+}-mediated oxidation of liposomes

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Abstract Heparin (HE) exhibited a protective effect on liposome peroxidation induced by Fe^{2+} and Cu^{2+}, decreasing the formation of both conjugated dienes and thiobarbituric acid reactive substances (TBARS) in a dose-dependent manner. The antioxidant activity was more relevant in the oxidizing system employing Fe^{2+} and H_2O_2 and generating the highly reactive $^{\cdot}OH$ radical. The analysis of liposome size distribution by quasi-elastic laser light scattering showed that: (1) the native structure of the particles was completely lost after exposure to Fenton reagent; (2) the presence of HE in the reaction mixture completely prevented the peroxidative damage on liposomes. Thus, HE acts as an antioxidant factor on membrane lipid bilayer. This suggests that HE, released from mast-cell granules during inflammatory processes, might locally protect the cell membrane from the oxidative injuries.

Key words: Heparin; Liposome; Copper oxidation; Iron oxidation

1. Introduction

Heparin is normally produced as a proteoglycan (PG) in connective tissue type mast cells and stored in their secretive granules [1,2]. The polysaccharide chains are then degraded by a specific endo-β-D-glucuronidase into fragments, which are commonly recognized as 'heparin' (HE) and released in response to specific stimuli, as in the case of inflammation [1,2]. The main effect of HE is to accelerate the inactivation of coagulation serine proteinases by the serpin antithrombin and HE cofactor II [3]. Recent findings suggest that HE may also play a protective role in oxidative stress. In fact, HE was found to inhibit the generation of conjugated dienes and thiobarbituric acid reactive substances (TBARS) when incubated with Fe^{2+} and γ-linolenic acid [4]. Moreover, an antioxidant effect of HE was shown on Cu^{2+}-mediated oxidation of human LDL [5].

On this basis, we studied whether the antioxidant effect of HE could be exerted also on experimental models of biological membranes, such as liposomes. We employed both Fe^{2+} and Cu^{2+} as oxidizing agents on the consideration that these transition metals are present in human body and are involved in oxidative processes [6]. After exposure of liposomes to oxidative stress, we monitored both primary and end products of lipid peroxidation. The structural consequences of peroxidative damage on the particles were evaluated determining liposome size distribution by quasi-elastic laser light scattering (QELLS) analysis.

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2. Materials and methods

2.1. Preparation of liposomes

Two different preparations of liposomes were obtained, according to Hope et al. [7] with minor modifications. An extruder (Lipex Biomembranes, Inc, Vancouver) equipped with two polycarbonate filters of 100 nm pore size (Costar) was used under nitrogen pressure of 25 bars at 40°C. The first preparation used phosphatidylcholine from fresh egg yolk and was employed in Fe^{2+}-induced oxidation and in the analysis of liposome size distribution. The second preparation used phosphatidylcholine from dried egg yolk and was employed in Cu^{2+}-induced oxidation to monitor conjugated-diene formation. The percentage fatty acid compositions of phosphatidylcholine from fresh and dried egg yolk, as determined by gas-chromatography assay [8], were as follows: phosphatidylcholine from fresh egg yolk: 16:0, 31 ± 0.4; 16:1, 1.6 ± 0.1; 18:0, 13.1 ± 0.8; 18:1, 31.8 ± 2.0; 18:2, 15.8 ± 0.8; 20:4, 3.3 ± 0.5; 22:6, 1.2 ± 0.3. Phosphatidylcholine from dried egg yolk: 16:0, 29.8 ± 0.9; 16:1, 1.7 ± 0.2; 18:0, 15.4 ± 0.4; 18:1, 30.2 ± 0.2; 18:2, 15.5 ± 0.3; 20:4, 6.0 ± 0.2; 22:6, 1.4 ± 0.2. The values are the mean ± S.D. of 4 determinations.

2.2. Measurement of lipid peroxidation

Lipid peroxidation was measured by determining both the primary (conjugated dienes) and the end products (malondialdehyde, MDA, and other β-aldehydes) of the process.

The formation of UV-absorbing conjugated dienes was monitored at 233 nm [9], after incubation of liposomes (17 μM as organic phosphate) with 100 μM $CuSO_4$ in 8 mM PBS at 25°C, in either the absence or presence of HE (ranging from 180 to 750 μg/ml and corresponding to 90–375 μg/ml of hexuronate, respectively). The reaction mixture contained a relatively low concentration of liposomes from dried egg yolk phosphatidylcholine, which had an almost 2-fold greater content of arachidonic acid than phosphatidylcholine from fresh egg yolk. Consequently, more bis-allylic hydrogens favouring peroxidative reaction were available. This favoured a more rapid formation of conjugated dienes, that was high enough to be monitored with time at 233 nm. In some experiments the liposome preparation was extracted with chloroform:methanol (2:1, v/v) before and after oxidation with $CuSO_4$. The absorbance spectrum in the UV of both extracts was then registered. The difference spectrum between the oxidized and unoxidized sample showed a K band near 233 nm, confirming that the increase in 233 nm absorption was imputable to conjugated-diene formation [10]. A secondary absorption maximum was also recorded, due to ketone dienes, in the region 260–280 nm [10]. Conjugated dienes were also assayed after Fe^{2+}-induced oxidation of liposomes. In this case, the assay was performed after methanol extraction to avoid interference by Fe^{2+} [11].

The end products of lipid peroxidation were assayed using the thiobarbituric acid (TBA) method after trichloroacetic acid precipitation [12]. In the case of Fe^{2+}-induced oxidation, the incubation mixtures contained 35 μl of liposome suspension (2.25 mM as organic phosphate), 115 μl of PBS containing different HE amounts and either 150 μl of water or 150 μl of Fenton reagent (2 mM H_2O_2, 1 mM $FeSO_4$). The samples were incubated at 40°C for 180 min. In the case of Cu^{2+}-induced oxidation, where a lower concentration of liposomes was used, higher sensitivity was required and TBARS concentration was determined fluorimetrically at 553 nm with excitation at 515 nm [12]. As we recently reported elsewhere [13], a direct HPLC determination of TBA-MDA complex and other TBARS produced after liposome peroxidation showed that the standard TBA test (using trichloroacetic acid supernatant) measured exclusively MDA in peroxidized liposomes under the used experimental conditions.

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No TBARS were formed when HE (at a concentration varying from 0.5 to 6 mg/ml, corresponding to 250–3000 μg/ml of hexuronate) was exposed to Fenton reagent in the absence of liposomes. Moreover, the presence of HE did not affect the quantitative determination of MDA in TBA test, indicating that under our experimental conditions TBARS were not bound to HE.

2.3. Liposome size distribution analysis

The assay was performed by QELLS [14], using a BI 90 particle sizer (Brookhaven Instrument Co, Holtsville, NY), equipped with a laser source at an excitation wavelength of 632.8 nm. The fluctuation in scattered light intensity generated by vesicle diffusion in solution was analysed by digital autocorrelation technique. The results came from 5–7 runs of 1000 cycles/run at 25°C. QELLS analysis of liposome size distribution requires relatively high concentration of particles to obtain a significant intensity of scattered light. Under our experimental conditions, only the Fe^{2+}-induced oxidation system afforded a liposome concentration high enough to permit QELLS analysis.

2.4. Analytical methods

Hexuronate was assayed by the carbazole method [15]. Total and inorganic phosphate was assayed as described by Ames [16].

3. Results

3.1. HE effect on Fe^{2+}-induced oxidation of liposomes

After 180 min incubation of liposomes with Fenton reagent, the concentration of conjugated dienes in the reaction mixture was 38.5 μM, whereas the concentration of TBARS was 6.5 μM. This ratio between the concentrations of primary products (conjugated dienes) and end products (TBARS) was comparable with that reported in similar experiments on lipid peroxidation [11]. The addition of HE strongly inhibited the formation of lipid peroxidation products. The inhibition of TBARS production increased progressively in the range of HE concentrations between 1 and 100 μg/ml, reaching completion at 100 μg/ml HE (Fig. 1). In our reaction mixture, the concentration of phosphatidylcholine was 260 μM (as organic phosphate) and the concentration of fatty acids with 3 or more double bonds (the main ones responsible for MDA formation) [6] was 11.7 μM, according to the fatty acid composition of fresh egg yolk phosphatidylcholine. Therefore, our free radical producing system induced the formation of 0.55 μmol of MDA/μmol of polyunsaturated fatty acid.

After incubation with Fenton reagent, HE was analysed by gel-filtration chromatography on Bio-Gel P6 and Bio-Gel P30 columns (140×1 cm, eluted with 0.5 M NaCl, flow rate 5.5 ml/h) and by cellulose acetate electrophoresis [17]. Both the hexuronate elution pattern and the electrophoretic mobility remained unchanged after the incubation, indicating that, under our experimental conditions, incubation with Fe^{2+}-H_2O_2 neither induced significant formation of small size oligosaccharide fragments nor affected the charge density.

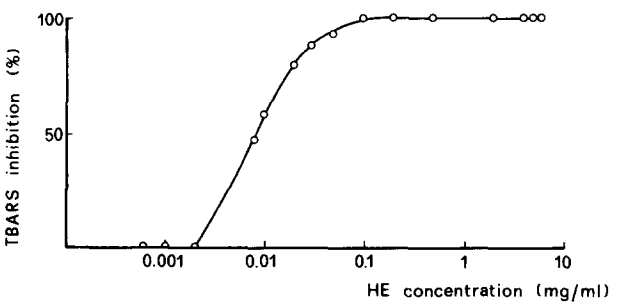

Fig. 1. HE effect on Fe^{2+}-induced oxidation of liposomes. Liposomes were incubated with Fenton reagent as described in section 2 and TBARS assayed as end products of lipid peroxidation. The inhibition of liposome peroxidation in the presence of different concentrations of HE was expressed as a percentage of TBARS formation with reference to a control incubation mixture with Fenton reagent only. Similar results were obtained in three independent experiments.

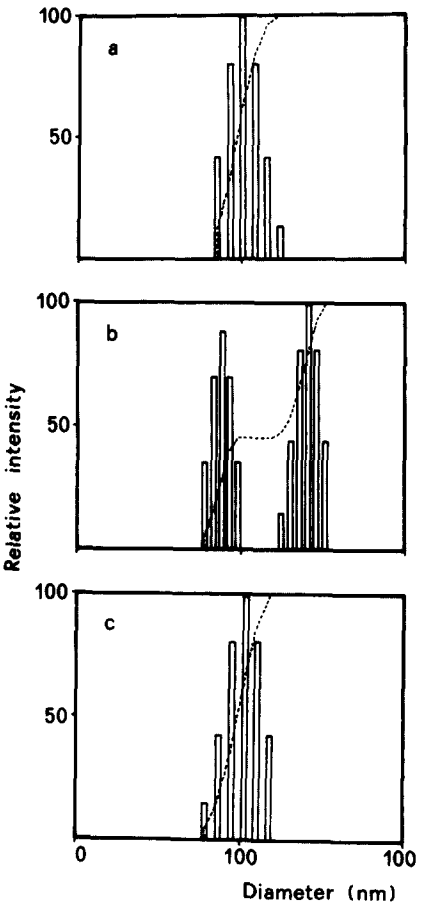

Fig. 2. QELLS analysis of liposome size distribution. (a) Control liposomes, incubated in the absence of Fenton reagent; mean diameter 107 nm. (b) Liposomes incubated with Fenton reagent. In this case, the particles were divided into two populations, showing smaller (77 nm) and larger (257 nm) mean diameter size. (c) Liposomes incubated with Fenton reagent in the presence of 3 mg/ml HE; mean diameter 107 nm. The columns indicate the intensity of the light scattered by the different liposome populations as percent of the maximum recorded value. The dotted line indicates the cumulative distribution.

The structural consequence of liposome peroxidation was evaluated by QELLS analysis of liposome size distribution. Fe^{2+}-induced oxidation changed the unimodal distribution of liposome size (mean diameter 107 nm, polydispersity 0.06 ± 0.005) (Fig. 2a) into a bimodal distribution, with smaller and larger populations (mean diameter size 77 and 257 nm, respectively, polydispersity 0.256 ± 0.008) (Fig. 2b). The low mean diameter population consisted of 90% of the liposomes on the basis of their total volume and of 99% of the liposomes on the basis of their total number. The bimodal distribution was probably due to the loss of the native structure of liposomes, mainly related to fragmentation of the particles and to simultaneous partial aggregation of the fragments. The presence of 3 mg/ml HE in the reaction mixture completely prevented the alterations induced by Fenton reagent, maintaining

the unimodal distribution of liposome size (mean diameter size 107 nm, polydispersity 0.116 ± 0.09) (Fig. 2c).

3.2. HE effect on Cu^{2+}-induced oxidation of liposomes

Fig. 3a shows that HE inhibited the formation of conjugated dienes in a dose-dependent manner, again suggesting an increase of liposome resistance to oxidation. In Cu^{2+}-induced liposome oxidation the protective effect of HE was detectable at concentrations over one order of magnitude greater than those effective in Fe^{2+}-induced oxidation. Therefore, conjugated-diene formation was monitored by recording the increase in A_{233} absorption with time in the presence of an effective concentration of HE of 180 and 750 μg/ml. Assuming a molar absorbance ε_{233nm} for conjugated dienes of 28 000 l mol^{-1} cm^{-1} [18], the amount of diene formation after 125 min incubation (expressed as μmol dienes/μmol organic phosphate) was 0.21 in the absence of HE, 0.16 in the presence of 180 μg/ml HE and 0.04 in the presence of 750 μg/ml HE.

The first derivative of diene formation versus time profiles was then calculated to obtain the change in oxidation rates as a function of time. The calculation was performed by computing the forward difference quotient [19] (Fig. 3b). The presence of HE lowered the value of the maximum velocity of lipid peroxidation and induced a more marked decrease in oxidation rates as a function of time. The percentage decrease in oxidation rate after 110 min was 67.7 in the absence of HE and 83.2 and 93.8 in the presence of 180 μg/ml and 750 μg/ml HE, respectively.

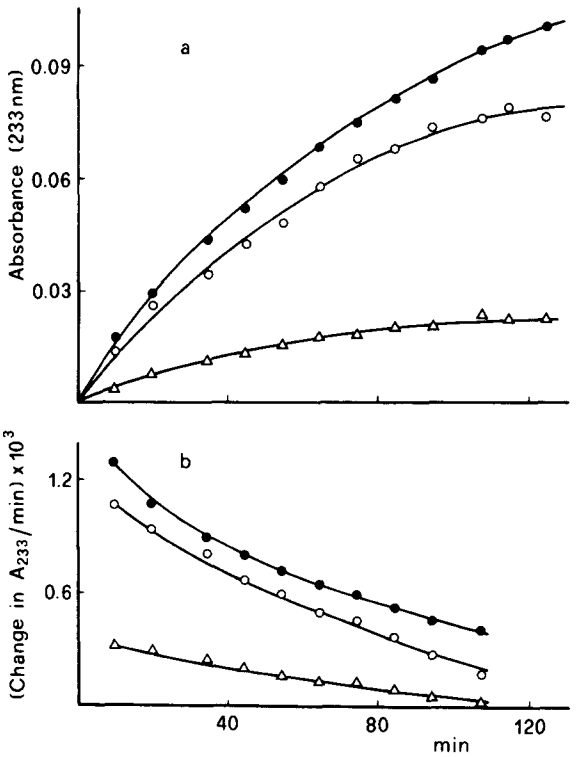

Fig. 3. HE effect on Cu^{2+}-induced oxidation of liposomes. (a) Diene versus time profiles, corrected for the initial absorption measured at time 0. Liposomes were incubated with 100 μM $CuSO_4$ as described in section 2, in the absence (●) and presence of 180 μg/ml HE (○) and 750 μg/ml HE (△). The formation of UV-absorbing conjugated dienes was followed recording the absorbance increase at 233 nm in 1 cm cuvette at 25°C. Similar results were obtained in three independent experiments. (b) First derivatives of diene versus time curves. Control liposomes, incubated in the presence of 100 μM $CuSO_4$ only (●); liposomes incubated in the presence of 100 μM $CuSO_4$ and 180 μg/ml HE (○); liposomes incubated in the presence of 100 μM $CuSO_4$ and 750 μg/ml HE (△). The data were smoothed by a moving average calculation (average change in 233 nm absorption over 20 min) to reduce the noise in the plot.

The protective effect of HE was also evident from a lower TBARS production, assayed fluorimetrically at the end of incubation. The concentrations of TBARS were: 0.19 μM in the absence of HE and 0.13 μM and 0.06 μM in the presence of 180 μg/ml and 750 μg/ml HE, respectively.

4. Discussion

Our study showed that HE inhibited the lipid peroxidation induced by Fe^{2+} and Cu^{2+} in a dose-dependent manner. As lipid substrate we used liposomes of phosphatidylcholine from egg yolk, which have been successfully employed to measure the antioxidant effect of a number of molecules [20]. We used two oxidizing systems (Fenton reagent and Cu^{2+}), that induce lipid peroxidation by different mechanisms: the former by the production of $^{\bullet}OH$ radical close to the lipid bilayer, the latter by the action of Cu^{2+} on preformed hydroperoxides. The reaction of Fe^{2+} and H_2O_2 is also a feasible source of $^{\bullet}OH$ in vivo, as H_2O_2 is generated in many aerobic cells and iron has been shown to catalyse radical reactions in biological fluids [21]. Cu^{2+} is commonly used to induce human plasma LDL oxidation [6] and may react with lipid hydroperoxides, promoting the formation of peroxyl radicals and propagating lipid peroxidation.

Under our experimental conditions, the protective effect of HE was significant in both the oxidizing systems, but was more relevant in the case of Fe^{2+}-induced oxidation, where lower HE concentrations were required for the antioxidant activity. These results could well depend on different protective mechanisms exerted by HE in our oxidizing systems. The present data do not indicate specific mechanisms for the HE antioxidant effect. We employed high concentrations of metal ions (100 μM Cu^{2+} and 500 μM Fe^{2+}) and this seems to rule out an effective chelation of the metals by HE. However, an HE interaction with phosphatidylcholine hydrophilic heads on the outer layer of liposomes might well cause a particle change less favourable to lipid peroxidation. It should be added that Fenton reagent produces primarily the highly reactive $^{\bullet}OH$ radical, which can be scavenged by HE. The bleaching of $^{\bullet}OH$ by HE would then explain the greater antioxidant effect of HE in comparison with that in Cu^{2+}-induced oxidation.

It is also notable that HE was effective at very low concentrations, the fall being of the same order of magnitude as those used in medical therapy.

The analysis of liposome size distribution by QELLS showed that the particles disintegrated following oxidative stress. The radical reaction on polyunsaturated fatty acids, which leads to TBARS production, results in chain fragmentation with the consequent macroscopic appearance of particle disintegration. The preventive antioxidant effect of HE was evident both on the formation of the chemical products of the peroxidation process and on the structural consequence of the oxidative damage of the particle.

These results are consistent with a possible protective role of HE in lipid peroxidation. This might occur in the course of inflammatory processes, where HE, released by mast-cell granules [1,2], could well protect the lipid component of cell membrane in the affected tissue compartment. It is note-

whorty that HE displays a high structural homology to heparan sulphate, which is present on the outer layer of cell membrane [22]. By inference, heparan sulphate (and other hexuronate containing glycosaminoglycans) could represent a protective covering for the cells against oxygen free radicals.

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