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Chondroitin 4-sulphate exhibits inhibitory effect during Cu^{2+}-mediated LDL oxidation

Riccardo Albertini[a,b,*], Pilar Ramos[a], Andreas Giessauf[a], Alberto Passi[c], Giancarlo De Luca[c], Hermann Esterbauer[a]

[a]*Institute of Biochemistry, University of Graz, Schubertstrasse 1, A-8010 Graz, Austria*
[b]*Laboratory of Clinical Chemistry, IRCCS Policlinico S. Matteo, University of Pavia, Piazzale Golgi, 27100 Pavia, Italy*
[c]*Department of Biochemistry, University of Pavia, Via Taramelli 3b, 27100 Pavia, Italy*

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Abstract Chondroitin 4-sulphate (C4S), a basic component of the extracellular matrix of the artery wall, inhibited copper-induced low density lipoprotein (LDL) oxidation by prolonging the lag time and reducing the rate of propagation. LDL oxidation was assessed by monitoring conjugated dienes and low level chemiluminescence. A possible initial key reaction in LDL oxidation, the reduction of copper(II) to copper(I) by LDL, was decreased in the presence of C4S. Moreover, C4S protected tryptophan residues of Apo-B-100 in the early stage of LDL oxidation and during the subsequent propagation phase. The presence of the sulphate group in position 4 of *N*-acetylgalactosaminyl residues of C4S is crucial for protective activity. In fact, the structurally related chondroitin 6-sulphate, also present in tissues, had no effect on LDL oxidation. These data suggest that C4S may contribute to protect LDL against oxidation in arterial intima.

Key words: Chondroitin sulfate; Glycosaminoglycan; Low density lipoprotein; Copper oxidation

1. Introduction

Several lines of evidence support the hypothesis that oxidation of low density lipoprotein (LDL) may play a crucial role in the initiation and progression of atherosclerosis. Oxidized LDL is taken up avidly by macrophages via scavenger receptors, leading to foam-cell formation and exhibits also chemotactic, cytotoxic and prothrombotic effects [1,2].

It is generally accepted that LDL oxidation may occur in arterial intima, which is at least partially segregated from plasma antioxidants and metal chelating proteins [1,2]. Therefore, the oxidative modification of LDL is likely to occur in close contact with proteoglycans, a basic component of the interfibrillar space. In vascular tissue the most abundant proteoglycan contains a large number of glycosaminoglycan (GAG) chains, which are mainly represented by chondroitin sulphate (CS) [3,4]. The possibility of a close interaction between LDL and GAG chains in the extracellular matrix of arterial intima is supported by the observation that such complexes may be formed in vitro [5,6] and can be extracted from arterial wall [7].

Following these considerations, we investigated whether CS might interfere with LDL oxidation. In tissues CS chains are prevalently sulphated either in position 4 (C4S) or in position 6 (C6S) of *N*-acetylgalactosaminyl residues [3]. Therefore, both polymers were considered in our study. We found that only C4S was able to inhibit Cu^{2+}-induced oxidation of LDL, indicating that the presence of sulphate groups on C-4 was required for the protective properties of the chain. The effect of C4S was studied with regard to kinetic parameters of LDL oxidation by Cu^{2+} and to tryptophan (Trp) residues of Apo-B-100, possibly involved in the initiation phase of LDL oxidation [8].

2. Materials and methods

2.1. Materials

2,2′-Azobis(2-amidinopropane hydrochloride) (AAPH) was purchased from Polysciences (Warrington, PA). C4S from whale cartilage and C6S from shark cartilage were obtained from Seikagaku (Tokyo, Japan). The sulphation pattern of the chains was checked by HPLC separation of unsaturated disaccharides obtained after chondroitinase ABC digestion [9]. The disaccharide compositions (in percentage) were: C4S, α-ΔUA-[1→3]-GalNAc-4S, 82.1; α-ΔUA-[1→3]-GalNAc-6S, 17.9; C6S, α-ΔUA-[1→3]-GalNAc-4S, 15; α-ΔUA-[1→3]-GalNAc-6S, 80; α-ΔUA-2S-[1→3]-GalNAc-4S, 5. Other reagents used were of AR grade, obtained from Merck (Darmstadt, Germany) or Sigma (St. Louis, MO).

2.2. Preparation of LDL

EDTA-plasma (1 mg/ml) was prepared from blood of normolipidemic, healthy volunteers of both sexes (age 25–35) after overnight fast. Plasma samples were pooled and frozen at −80°C in 0.6% sucrose for up to 4 weeks. LDL was prepared by ultracentrifugation (Beckman L70) in a single step discontinuous gradient using an NVT 65 rotor (Beckman)[10]. LDL was stored in a vial under argon at 4°C, in the dark and used within 1 week after preparation. LDL chemical composition was normal according to [2].

2.3. LDL oxidation

Before oxidation, LDL was freed of EDTA by gel filtration with an Econo-Pac 10 DG column (BioRad, Hercules, CA) using phosphate-buffered saline (PBS) as eluent. The composition of PBS was: 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4. LDL concentration in PBS was determined by total cholesterol assay (CHOD-PAP kit, Boehringer Mannheim, Germany). Assuming a molecular mass of 2.5 MDa for LDL and a cholesterol content of 31.6%, a concentration of 0.1 μM LDL corresponded to 0.25 mg/ml total mass and 79 μg/ml cholesterol [10].

0.1 μM LDL, freed of EDTA, was oxidized with $CuSO_4$ at 37°C in PBS in the absence and presence of CS. Oxidation was followed by monitoring the increase in absorbance at 234 nm (A_{234}), due to the formation of conjugated dienes (CD) from polyunsaturated fatty acids [11]. Lag time, maximal rate of propagation and CD concentration

*Corresponding author. Fax: (43) (316) 3809845.

Abbreviations: LDL, low density lipoprotein; CS, chondroitin sulphate; C4S, chondroitin 4-sulphate; C6S, chondroitin 6-sulphate; AAPH, 2,2′-azobis(2-amidinopropane hydrochloride); PBS, phosphate-buffered saline; GAG glycosaminoglycan; Trp, tryptophan; CD, conjugated diene; ΔUA, unsaturated uronic acid; GalNAc-4S, *N*-acetylgalactosamine 4-sulphate; GalNAc-6S, *N*-acetylgalactosamine 6-sulphate.

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were considered as indices of LDL oxidation and determined according to [10,11].

Alternatively, 0.1 μM LDL, freed of EDTA, was oxidized with AAPH in PBS at 37°C. The oxidation was then monitored by recording the increase of A_{234}.

2.4. Chemiluminescence measurements

Low level chemiluminescence was measured in a Lucy 1 luminometer (Anthos Labtec Instruments, Salzburg, Austria) equipped with a photomultiplier tube (sensitivity ranging from 300 to 700 nm). Integration time for each data point was set to 60 s. 0.3 μM LDL in PBS was oxidized with $CuSO_4$ in the presence of various concentrations of CS. The assays were performed at 37°C in a white microplate.

2.5. Measurement of LDL-Trp fluorescence

Trp fluorescence was measured in a solution of 0.1 μM LDL in PBS at 37°C, using a Shimadzu RF 5001 PC fluorometer (emission 331 nm, excitation 282 nm)[8]. The kinetics of LDL oxidation was followed by measuring the decrease of Trp fluorescence, corresponding to the decomposition of this amino acid, after addition of 1.6 μM Cu^{2+}, in the absence or presence of CS. The quenching of Trp fluorescence was calculated as percent decrease of LDL-Trp fluorescence measured 2 min after Cu^{2+} addition (1.6 μM, final concentration).

2.6. Assay of Cu^{2+} reduction

The ability of LDL to reduce Cu^{2+} was assayed using bathocuproine, a selective chelator of Cu^{+} [12]. 0.1 μM LDL in PBS was incubated with 360 μM bathocuproine at 37°C, in the presence or absence of CS. Cu^{+} concentration was calculated using a calibration curve obtained after Cu^{2+} reduction by 1 mM ascorbate.

3. Results

Under our experimental conditions, the profile of CD vs. time showed three evident phases, a lag phase, a propagation phase and a decomposition phase in agreement with a previous report [10] (Fig. 1). The addition of C4S at concentrations ranging from 0.2 to 2 mg/ml resulted in a dose-dependent increase of lag phase (Fig. 1). In contrast, C6S was completely ineffective at concentrations up to 2 mg/ml (Fig. 1, inset). The plot of lag time vs. C4S concentration showed a linear relationship (Fig. 2A). C4S also reduced the maximum rate of LDL oxidation (V_{max}), expressed as ΔA_{234}/min; the plot of rate against C4S concentration resulted in a non-linear relationship (Fig. 2B). The different effect of C4S on lag time and V_{max} might depend on the different chemical processes operating during the lag phase and propagation phase. C4S was not able to modify the value of total CD production at the end of the propagation phase, which was 24 ± 1 μM, indicating a high level of oxidation (Fig. 1). The ability of C4S to prolong the lag time and to reduce V_{max} showed that it was effective as an inhibitor of the oxidation process.

The protective effect of C4S was confirmed when the oxidation of LDL was assayed by monitoring chemiluminescence associated with the lipid peroxidation process [13]. As in the case of the CD profile, the time course of chemiluminescence was characterized by a lag phase and by a phase with a rapid increase in light emission (Fig. 3A,B). Addition of C4S in the incubation mixture induced prolongation of the lag phase (Fig. 3A,B). C6S was again ineffective, as shown in the CD assay.

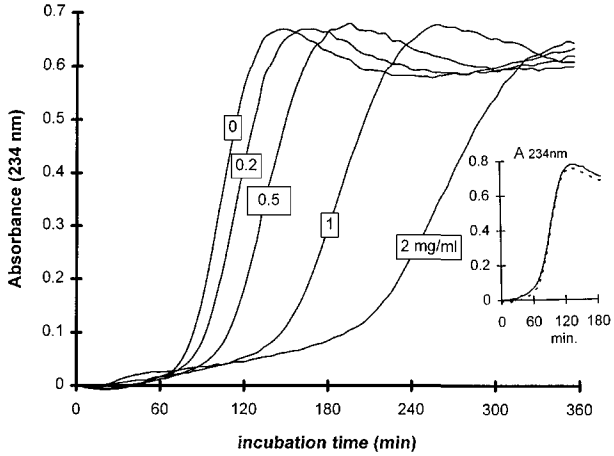

Fig. 1. C4S effect on the kinetics of copper-induced LDL oxidation. 0.1 μM LDL in 10 mM PBS was incubated with 1.6 μM Cu^{2+} in the absence and presence of increasing C4S concentrations, as indicated. CD formation was followed by recording the increase in absorbance at 234 nm at 37°C. CD concentration was calculated from the A_{234} using a molar absorbance of 29 500 l mol^{-1}. Experiments performed with copper concentrations ranging from 0.8 to 4 μM confirmed the ability of C4S to prolong the lag phase. Inset: C6S effect on the kinetics of copper-induced LDL oxidation. LDL was oxidized in the absence (continuous line) and presence of C6S concentrations up to 2 mg/ml (dotted line).

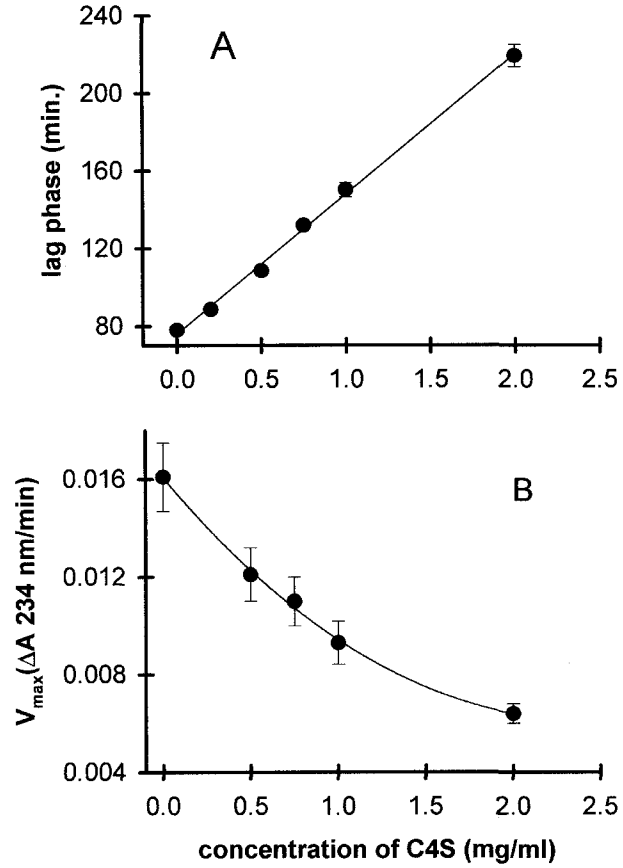

Fig. 2. (A) C4S effect on lag time during copper-induced LDL oxidation. Lag time was graphically determined from CD vs. time profiles of Fig. 1A, as the intercept of the tangents to the slow and fast increase of diene absorption [11]. Values are means ± S.D. of 4 different experiments. The line was drawn by linear regression analysis. (B) C4S effect on maximum rate of propagation during copper-mediated LDL oxidation. Maximum rate of propagation (V_{max}) was expressed as ΔA_{234}/min and was determined as the peak value of the first derivative of CD vs. time profiles reported in Fig. 1 [11]. Values are means ± S.D. of 4 different experiments. The line was drawn by non-linear regression analysis.

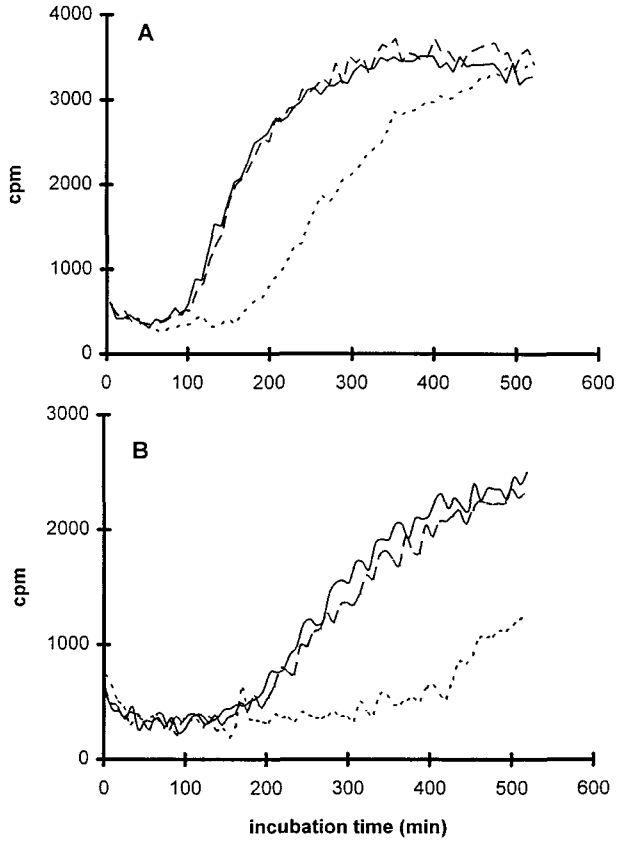

Fig. 3. Monitoring of LDL oxidation by low-level chemiluminescence. The emission of light is expressed in counts per min (cpm). 0.3 μM LDL was incubated at 37°C in the absence (continuous line) and presence of 1 mg/ml C4S (dotted line) and 1 mg/ml C6S (dashed line) at Cu^{2+} concentration of 1.48 μM (A) and 0.74 μM (B). Experiments performed with different copper concentrations (ranging from 0.74 to 4 μM Cu^{2+}) confirmed the protective role of C4S and the inefficiency of C6S.

The effect of CS on some indices of the protein moiety of LDL during oxidation was also investigated. The degradation of a proportion of Trp residues of Apo-B-100 is an early event during Cu^{2+}-mediated oxidation of LDL, resulting from a lipid peroxidation independent process [8]. The addition of 1.6 μM Cu^{2+} caused a quenching effect on Trp fluorescence (resulting from Cu^{2+} complexation by Trp residues) of 11% in the control and of 5.6% in the presence of C4S (Fig. 4A). Since Apo-B-100 contains 37 Trp residues, each proportionally contributing to the total fluorescence emission, it can be deduced that about 4 Trp residues for the LDL particle are immediately accessible to copper ions; this value is reduced to 1.7 in the presence of C4S. This observation suggests that C4S protects Trp residues probably involved in the initiation process. In contrast, C6S had no influence on Trp quenching by copper (Fig. 4A).

The loss of Trp fluorescence during LDL oxidation was monitored with increasing time (Fig. 4B). We identified a lag phase, characterized by a low decrease in fluorescence emission, that was substituted by a fast decrease (presumably resulting from interaction of Trp with oxidizing lipids) after about 80 min in the control. The addition of C4S prolonged the lag phase of Trp destruction to 150 min, whereas C6S was not able to modify the time course of Trp fluorescence loss (Fig. 4B).

It has been suggested that the reduction of Cu^{2+} to Cu^{+} might represent a rate-limiting step of LDL oxidation [2]. The formation of Cu^{+} by LDL, in the presence and absence of CS, was assayed according to [12]. The addition of 2 mg/ml C4S to an incubation mixture containing 0.1 μM LDL and 30 μM Cu^{2+} induced the following effects: (1) it reduced the amount of Cu^{+} formed after 10 min of incubation from 14.5 ± 0.5 to 10 ± 0.4 μM; and (2) it decreased the maximum rate of Cu^{+} formation from 2 ± 0.03 to 1.3 ± 0.03 μM/min. These data indicate that C4S reduced the formation of Cu^{+}, that is generally considered as the prooxidant form of copper. In contrast, C6S did not affect Cu^{2+} reduction by LDL.

The formation of a stable redox inactive complex between C4S and copper might be a possible molecular mechanism responsible for the protective effect of C4S on LDL oxidation. To test this hypothesis, we used the experimental approach that is based on the degradation of ascorbic acid by Cu^{2+} [14]. In this reaction, ascorbic acid is both the reductant of Cu^{2+} and the target of Cu^{+}. C4S did not inhibit the degradation of

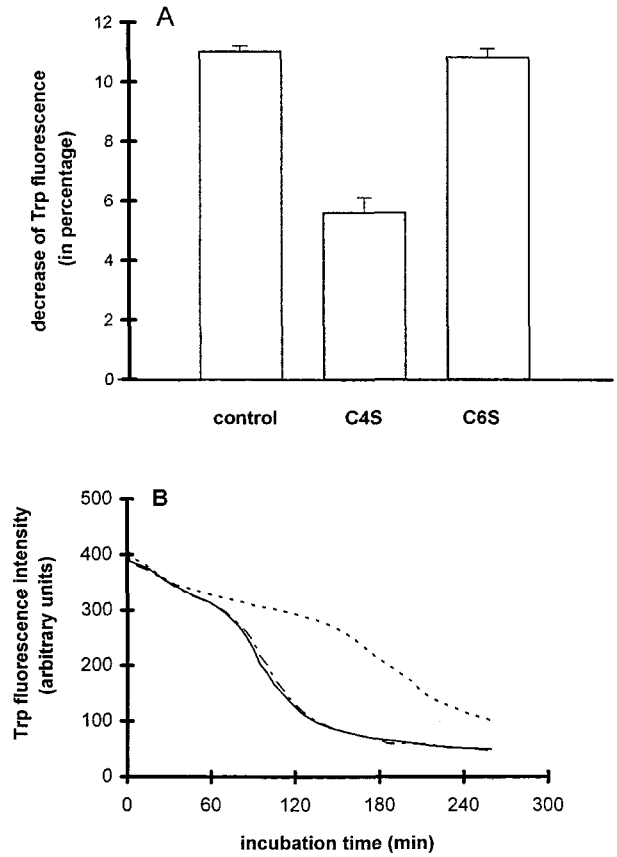

Fig. 4. (A) Effect of chondroitin sulphate on copper-induced Trp quenching. Trp fluorescence (excitation, 282 nm; emission, 331 nm) of 0.1 μM LDL in PBS was measured before and 2 min after addition of 1.6 μM Cu^{2+} in PBS at 37°C, in the absence (control) and presence of 1 mg/ml of C4S and C6S. The drop in fluorescence is expressed as percent decrease. (B) Kinetics of Trp fluorescence decrease during copper-mediated oxidation of LDL. LDL was oxidized in the absence (continuous line) and presence of C4S (dotted line) and C6S (dashed line). Experimental conditions were the same as described in (A).

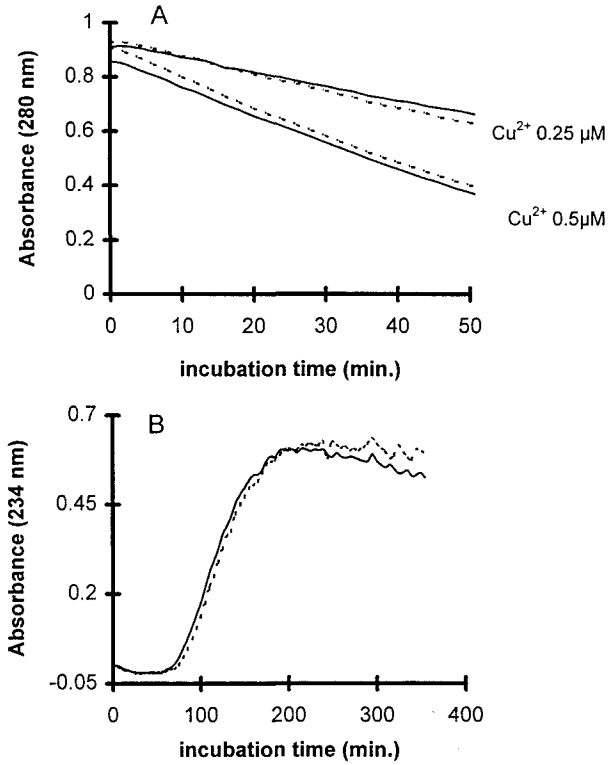

Fig. 5. (A) Effect of C4S on copper-catalyzed oxidation of ascorbate. 0.1 mM ascorbate was incubated in PBS at 37°C with Cu^{2+} (0.25 and 0.5 μM) in the absence (continuous line) or presence of 1 mg/ml C4S (dotted line). Ascorbate oxidation was monitored by the decrease of A_2 . (B) Effect of C4S on AAPH-induced LDL oxidation. 0.1 μM LDL was incubated with 1 mM AAPH at 37°C in PBS in the absence (continuous line) and presence of 1 mg/ml C4S (dotted line). LDL oxidation was followed by monitoring CD formation as the increase in A_{234}. Experiments performed with different AAPH concentrations (ranging from 1 to 3 mM) gave similar results.

0.1 mM ascorbic acid, indicating that it did not form redox inactive complexes with copper ions (Fig. 5A).

Finally, the possibility that C4S may also modulate the oxidation of LDL induced by a metal independent agent was investigated. LDL was exposed to AAPH, a generator of aqueous peroxyl radicals [2]. C4S did not affect the CD vs. time profile, indicating that it was ineffective against peroxyl-mediated LDL oxidation (Fig. 5B).

4. Discussion

Our results demonstrate that C4S exhibited a protective role during in vitro LDL oxidation by copper ions, whereas it was ineffective in a model of metal independent oxidation. In contrast, C6S lacked any effect on all the considered indices of LDL oxidation.

The sulphation pattern of CS in tissues is the result of a controlled metabolic activity [15] and involves important biological implications. In particular, in human aorta the relative content of C4S varies with age; the C4S/C6S ratio decreases from approx. 1 in young individuals (4 months to 5 years age) to 0.5–0.4 in adult age [16]. The loss of C4S and concomitant increase of C6S is even more pronounced in atherosclerotic lesions (C4S/C6S ratio 0.2 ± 0.05) [16]. Our results suggest that the modification of GAG content induced by age and atherosclerosis, consisting of a decrease of C4S with protective activity, may increase the susceptibility of LDL to oxidation. Moreover, the proportion of C4S in different vascular districts (pulmonary artery, iliac artery, aorta) decreases in an order corresponding to increasing risk for atherosclerosis [17]; this may at least partially depend on the different ability of the two isomers to protect LDL against metal dependent oxidative stress. The possible biologic relevance of our study is supported by the use of CS at concentration comparable to that found in vascular tissue [4,16] and by the fact that copper ions are possibly involved to a certain extent in LDL oxidation in vivo [18].

The decrease of Trp quenching by Cu^{2+} and the lowering of Cu^{+} formation indicate that C4S is effective in the early stage of LDL oxidation. These data, as well as the increase in the lag phase, suggest a reduction of the initiation rate in the presence of C4S. Radical scavenging properties of C4S are unlikely to be involved, as it was unable to inhibit peroxyl-mediated LDL oxidation.

According to several authors [2,8,14], part of the copper binding sites involved in the initiation of LDL oxidation might be located on Apo-B-100. Therefore, it seems reasonable to admit that a specific conformation of Apo-B-100 is important during the initiation phase. Under experimental conditions comparable to ours, an ionic interaction between the positively charged amino acids of Apo-B-100 and negatively charged GAGs has been described and shown to be dependent on their sulphation pattern [5,6]. Following these considerations, we suggest that C4S may interfere with LDL oxidation by covering or masking some copper binding sites on Apo-B-100, as a result of its interaction with the particle. C6S may be unable to realize a similar interaction, owing to the different position of the sulphate group, outside the sugar ring.

Finally, our results are compatible with the hypothesis that C4S and, possibly, other GAGs, may form a diffuse net in the extracellular matrix and in the pericellular environment, contributing to the protection of lipoproteins moving from intravascular compartment to arterial wall against some types of oxidative damage.

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