Kinetics of apolipoprotein E isoforms-binding to the major glycosaminoglycans of the extracellular matrix

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Abstract Apolipoprotein E (apoE), a key lipid transport protein, displays a heparin-binding property that is critical in several apoE functions. The kinetics of the interaction between apoE isoforms and glycosaminoglycans (GAGs) were studied using surface plasmon resonance. The dissociation constant of equilibrium K_D for apoE3-heparin interaction was estimated to be 12 nM for apoE3 and three common apoE isoforms revealed similar affinities for heparin. ApoE binds to GAGs in the following order: heparin > heparan sulfate > dermatan sulfate > chondroitin sulfate. The affinity parameter of the binding of low molecular weight heparins to apoE is correlated with the chain length. The effective number Z of electrostatic interactions between plasma apoE3 and heparin was assessed to be three. Metal chelators were able to diminish apoE-binding to heparin, suggesting some stabilizing effect of metal ions while reconstitution with lipids did not affect binding affinities for heparin, suggesting that the N-terminal heparin-binding site is responsible for apoE-containing lipoprotein interactions with heparin.

Key words: Apolipoprotein E; Heparin; Heparan sulfate; Dermatan sulfate; Chondroitin sulfate; Protein-binding

1. Introduction

Apolipoprotein E (apoE) is an important lipid transport protein in human plasma and brain. It serves as ligand for several types of receptors, including low density lipoprotein (LDL) receptor and LDL receptor-related protein (LRP) [1,2]. ApoE also binds to heparin and contains two sites involved in heparin-binding: a high affinity binding site in the N-terminal domain and a low affinity binding site in the C-terminal domain [3,4]. The heparin-binding property of apoE plays an important role in the sequestration step of the heparan sulfate proteoglycan (HSPG)/LRP metabolic pathway as well as in the independent HSPG pathway [5]. Interactions between lipoproteins and proteoglycans are related to the pathogenesis of atherosclerosis [6,7]. Heparin-binding activity of apoE was also proposed to be responsible for the inhibitory role of apoE in cell growth [8]. The human apoE gene has three common alleles, ε2, ε3 and ε4. Allele ε4 has been shown to be one of the major genetic risk factors of late-onset familial and sporadic Alzheimer's disease [9,10] and the mechanism that involves HSPG was hypothesized [5].

Thus, substantial evidence has been obtained for involving the heparin-binding activity of apoE in lipid metabolism, modulation of cell proliferation and atherogenesis. Studies of the interaction between apoE and extracellular matrix are particularly important for better understanding the mechanism of atherosclerosis considering the high physiological level of glycosaminoglycans (GAGs) in the vessel wall. In the present work, using surface plasmon resonance (SPR) biomolecular interaction analysis (BIA) technology, we studied and characterized the kinetics of apoE-GAG interactions. SPR is a biosensor technology that allows us to characterize the kinetics of bio-specific interactions under label-free conditions [11,12].

2. Materials and methods

2.1. Materials and proteins

Sensor chips SA were obtained from Biacore (Sweden). Native heparin, 3 and 6 kDa low molecular weight (LMW) heparins and heparan sulfate were obtained from Sigma (USA). Dermatan sulfate and chondroitin sulfate were purchased from Bioberica, S.A. (Spain). Heparin pentasaccharide was obtained from Sanofi (France).

2.2. Purification of proteins

ApoE3 was purified from plasma very low density lipoprotein (VLDL) of individuals with an ε3/3 genotype as described [13]. Recombinant isoforms apoE2, apoE3 and apoE4 were purified from *Escherichia coli* as described elsewhere [14]. Purified proteins showed a single band on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate [15]. Discoidal apoE/dipalmitoylphosphatidylcholine (DPPC) complexes were prepared by a detergent reconstitution method adapted from [16].

2.3. Biotinylation of GAGs

Heparin, heparan sulfate, dermatan sulfate and chondroitin sulfate were biotinylated using Biotin-LC-NHS (Pierce, USA) [17]. Five mg of GAG was dissolved in 2 ml of 50 mM bicarbonate buffer, pH 8.5. Biotinylation was performed in the presence of Biotin-LC-NHS predissolved in dimethylformamide at a 6:1 GAG:biotin molar ratio to exclude over-labelling. The mixture was incubated during 1 h at room temperature, unreacted biotin was removed and the buffer was changed to 25 mM Tris, pH 8.3, by centrifugation on a Centricon-3 (Amicon, USA).

2.4. Immobilization of ligands on sensor chips

SPR experiments were performed on a BIAcore X apparatus (Pharmacia Biosensor, Sweden). Biotinylated GAG was immobilized on a streptavidin-pre-coated SA sensor chip by its injection at a concentration of 0.25 mg/ml and at a flow rate of 5 μl/min to reach a saturation of surface.

2.5. SPR experiments

Binding experiments were performed at 25°C and at a flow rate of 5 μl/min as previously described [18]. In most of the experiments, the

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Abbreviations: apo, apolipoprotein; BIA, biomolecular interaction analysis; DETAPAC, diethylenetriaminepentaacetic acid; DPPC, dipalmitoylphosphatidylcholine; GAG, glycosaminoglycan; EDTA, ethylenediaminetetraacetic acid; HSPG, heparan sulfate proteoglycan; LDL, low density lipoprotein; LMW heparin, low molecular weight heparin; LRP, LDL receptor-related protein; PBS, phosphate-buffered saline; SPR, surface plasmon resonance; VLDL, very low density lipoprotein

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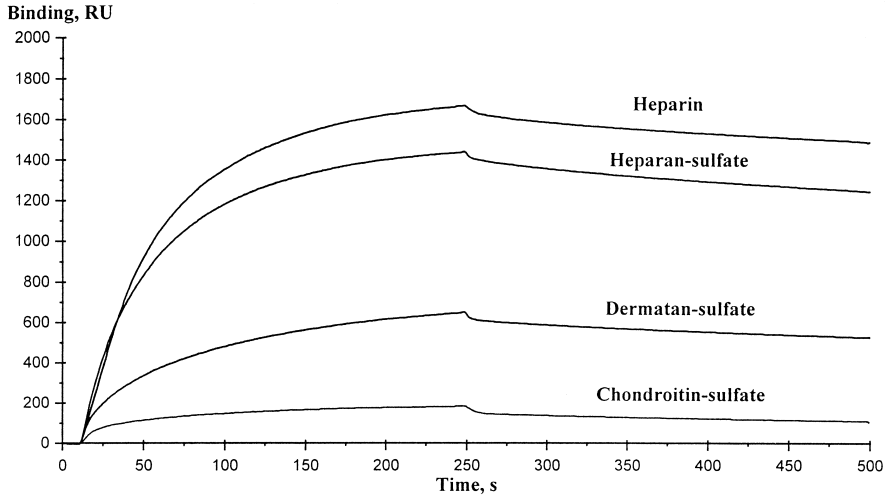

Fig. 1. Binding of human apoE3 to GAGs. Biotinylated GAGs were immobilized on SA sensor chip. Forty μl of apoE3 was injected over the sensor chip in 10 mM phosphate-buffered saline (PBS), pH 7.4. SPR analysis was performed at a flow rate of 5 μl/min at 25°C. Shown traces correspond to a apoE concentration of 1.0 μM.

running buffer was 10 mM sodium phosphate, 0.15 M NaCl, pH 7.4. To study the binding of proteins, 20 μl of analyte in the running buffer was injected over the GAG-coated sensor chip for the association phase (contact time 4 min). After 4 min of dissociation, surface was regenerated by injection of 10 μl of 1.0 M NaCl in phosphate buffer or 5 μl of 0.1% sodium dodecyl sulfate. Dissociation and association rates k_{diss} and k_{ass} were calculated by analysis of sensorgrams with BIAevaluation 2.2.4 software (Biacore, Sweden), using initial stages of dissociation and association phases, respectively, in accordance to the manufacturer recommendations. Equilibrium dissociation constant K_D was calculated as the ratio of k_{diss}/k_{ass}.

2.6. Other analytical procedures

The protein concentration was measured by the method of Lowry [19], using bovine serum albumin as the standard.

3. Results

GAGs were immobilized on the surface of a sensor chip using the streptavidin-biotin system. ApoE was consequently injected over the immobilized ligand at different concentrations to estimate rate constants and affinity parameters of apoE-GAG interactions. We demonstrated that apoE binds to all studied GAGs (Fig. 1). Among studied GAGs, apoE demonstrated the highest binding to heparin, whereas its binding to chondroitin sulfate was the lowest. The following order of apoE-binding was observed: heparin > heparan sulfate > dermatan sulfate > chondroitin sulfate. The differences found between GAGs in the binding to apoE were particularly due to increased dissociation of the protein from dermatan sulfate and chondroitin sulfate (Table 1).

Pre-incubation of apoE with GAG inhibited its binding activity, revealed by following injection over heparin- or heparan sulfate-coated sensor chip. The same order of GAG-binding affinities towards apoE was demonstrated by competition with heparin (Fig. 2A) and heparan sulfate (data not shown). Heparin in an equimolar ratio (1:1) completely blocked binding of apoE to heparan sulfate.

Furthermore, we investigated possible isoform-specific interactions of apoE to heparin. The heparin-binding property of recombinant apoE3 was actually equal to apoE3 isolated from plasma with a K_D of 13 and 12 nM, respectively. Three common apoE isoforms, apoE2, apoE3 and apoE4, demonstrated a similar affinity parameter of the binding with an equilibrium dissociation constant K_D of 15.6 ± 1.8, 13.4 ± 2.6 and 12.2 ± 3.0 nM, respectively ($n = 6$).

Effects of the heparin chain length on its interaction with apoE were studied in competition experiments. Binding of apoE to heparin-coated surface was reduced by pre-incubation of the protein with free fragments of heparin. Both 6 and 3 kDa LMW heparins as well as pentamer showed this effect. The affinity parameter of the fragments negatively correlated with the chain length (Fig. 2B). Data demonstrate that the pentamer still has a sufficient number of monomers to be able to interact with the protein.

The interaction between apoE and heparin is highly electrostatic and depends on the salt concentration [20]. In accordance to the molecular theory of polyelectrolyte solutions, the reaction between the protein and polyelectrolyte chain may be described as a ion-exchange process [21]. In the frames of the theory, the effect of the concentration of a monovalent cation on protein-polyelectrolyte interaction is described by the following equation:

$$\log K_D = \log K_{(0)} - Z\psi \log [M^+]$$

where K_D is the equilibrium dissociation constant of the bind-

Table 1
Binding of human plasma apoE3 to GAGs

GAG	k_{ass}, M^{-1} s^{-1}	k_{diss}, s^{-1}	K_D, nM
Heparin	$1.90\times10^{-4} \pm 0.65\times10^{-4}$	$2.19\times10^{4} \pm 0.53\times10^{4}$	11.5
Heparan sulfate	$2.36\times10^{-4} \pm 0.35\times10^{-4}$	$2.90\times10^{4} \pm 0.86\times10^{4}$	12.3
Dermatan sulfate	$2.28\times10^{-4} \pm 0.22\times10^{-4}$	$4.58\times10^{4} \pm 0.87\times10^{4}$	20.1
Chondroitin sulfate	$2.02\times10^{-4} \pm 0.42\times10^{-4}$	$6.12\times10^{4} \pm 0.14\times10^{4}$	30.3

Mean ± S.D. are shown ($n = 6$).

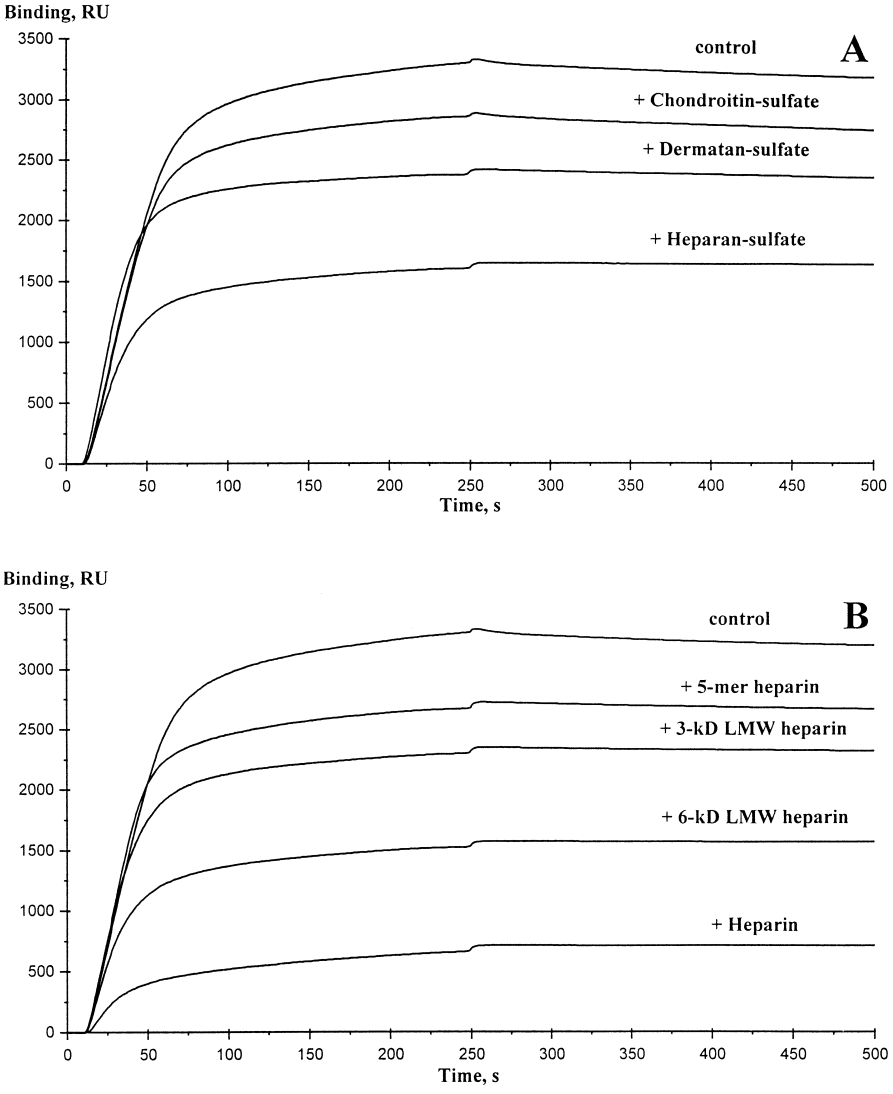

Fig. 2. Competition of GAGs (A) and LMW heparins (B) with native heparin for the binding to apoE. Native heparin was immobilized on SA sensor chip. ApoE was pre-incubated with an equimolar concentration of heparin competitor in 10 mM PBS and 40 μl of the mixture was injected over the heparin-coated surface at a flow rate of 5 μl/min. Shown traces correspond to an apoE concentration of 1.0 μM.

ing, $K_{(0)}$ is a constant reflecting the contribution of non-electrostatic interaction, Z is a number of ionic interactions, ψ is the axial charge density of polyelectrolyte, $[M^+]$ is the concentration of a monovalent ion. The affinity of apoE3-heparin interaction was determined as a function of the salt concentration. We found a linear dependency of log K_D on log $[M^+]$ (Fig. 3) and the slope was calculated as 2.2. Considering the axial charge density of heparin to be $\psi = 0.7$ [22], the number of ionic interactions between apoE and heparin was determined to be 3.1.

We then studied the effects of Ca^{2+} on apoE-binding to heparin. The presence of 1 mM $CaCl_2$ had no effects on the interaction (Fig. 4). In contrast, the addition of 1 mM ethylenediaminetetraacetic acid (EDTA) reduced the binding particularly due to increasing the dissociation rate. Similar effects were observed after the addition of another metal chelator, diethylenetriaminepentaacetic acid (DETAPAC).

To study the effects of lipid on the apoE-binding activity, the protein was reconstituted with DPPC to form discoidal apoE-lipid particles. Preliminary experiments showed that to

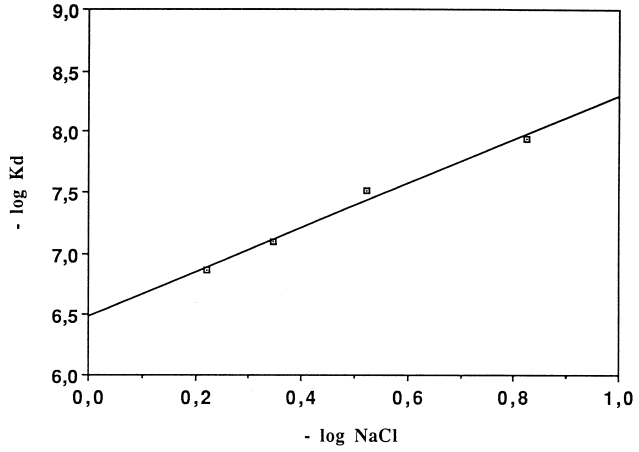

Fig. 3. Dependence of the K_D of apoE3-binding to heparin on the salt concentration. The dissociation constant of equilibrium was measured in 10 mM phosphate buffer, pH 7.4, and a varied salt concentration.

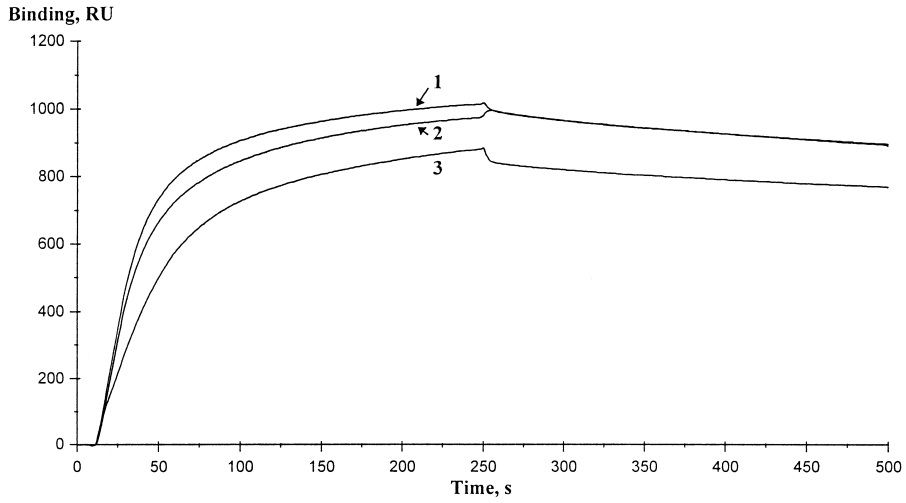

Fig. 4. Effects of Ca^{2+} and EDTA on apoE-binding to heparin. Heparin was immobilized on SA sensor chip. An apoE-binding assay was performed in 10 mM Tris-HCl, pH 7.4 (curve 1), in the presence of 1 mM $CaCl_2$ (curve 2) or 1 mM EDTA (curve 3). Traces shown correspond to injections of apoE3 at a concentration of 1.0 μM.

obviate mass transport limitations, the flow rate had to be increased to 30 μl/min and the binding of apoE/DPPC complexes to heparin was studied under these conditions (Fig. 5). The equilibrium dissociation constant K_D of apoE3/DPPC-binding to heparin was calculated to be 7 nM and was close to that of free apoE.

4. Discussion

Recent studies increasingly implicate proteoglycans as scaffold structures designed to accommodate proteins through non-covalent binding to their GAG side chains [23]. Binding to proteoglycans, particularly to HSPG, is a prerequisite to reveal their biological or pathophysiological activities for several important proteins of lipid metabolism, including lipoprotein lipase, hepatic lipase, apoB and apoE [5,24,25]. Some HSPGs, syndecans and perlecans, are also able to directly mediate the internalization of lipoproteins [26].

In the present work, we characterized the kinetics of apoE interaction with heparin and major GAGs of the extracellular matrix. We measured the dissociation constant of equilibrium of human apoE3 interaction with heparin K_D as 12 nM. Other common apoE isoforms showed close affinities. Apparently, the binding to heparin does not depend on some variability of apoE conformation likely due to flexibility of the GAG chain. Comparison of different GAGs revealed the following order of binding affinities for apoE: heparin > heparan sulfate > dermatan sulfate > chondroitin sulfate. Observed differences may be explained by the higher dissociation rate of dermatan sulfate and chondroitin sulfate from apoE. Apparently, these GAGs lack charged groups that stabilize the apoE-ligand complex as compared to highly sulfated heparin and heparan sulfate chains. Earlier, it has been demonstrated that extensively sulfated GAGs bind apoE-containing emulsions with high affinity in contrast to low-sulfated dermatan sulfate and chondroitin sulfate [27]. A heparin/heparinase-releasable pool of apoE on the HepG2 cell surface has previously been described [28,29]. Recently, chondroitin sulfate proteoglycans were shown to be the main site of apoE cell surface pool deposition in these cells along with HSPG [30].

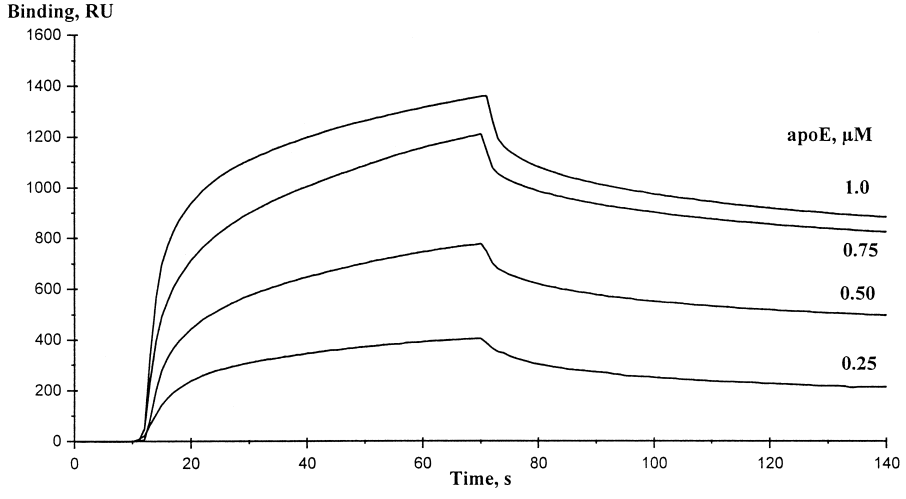

Fig. 5. Effects of lipid on apoE-binding to heparin. An apoE-binding assay was performed in 10 mM PBS, pH 7.4. Thirty μl of apoE3/DPPC complex preparation was injected at a flow rate of 30 μl/min over heparin-coated sensor chip. Concentrations of apoE were varied from 1.0 to 0.25 μM as shown to the right of traces.

Our data suggest that all three major GAGs of the extracellular matrix should be able to detain apoE on the cell surface. However, the pathophysiological importance of apoE-binding to dermatan sulfate and chondroitin sulfate still remains to be elucidated.

Furthermore, we estimated the ability of short-chain heparins to compete with heparin for apoE-binding. Despite diminished binding to apoE, LMW heparins were able to inhibit apoE interaction with native heparin. Pentamer demonstrated the lowest affinity among studied heparin fragments. It has generally been assumed that the major interactions between proteins and GAGs are electrostatic and involve the negatively charged sulfates and carboxylates on the GAG and positively charged residues on the protein [20]. The number of ionic interactions between apoE and heparin was calculated to be about three. Earlier, the number of electrostatic forces between lipoprotein lipase and heparin was determined to be about 10 [31]. This difference likely explains a higher affinity of the lipoprotein lipase-heparan sulfate interaction with $K_D = 0.3$ nM as compared to apoE. For example, addition of lipoprotein lipase to apoE-containing β-VLDL increases lipoprotein-binding affinity for HSPG 12–55-fold [32].

It has been proposed that apoE can bind metal ions as the protein is able to modulate the kinetics of the metal-induced lipoprotein oxidation and aggregation of β-amyloid [33,34]. Moreover, it was demonstrated on HepG2 cells that EDTA or ethylenebis(oxyethylenenitrilo)tetraacetic acid are able to displace part of the cell surface-associated pool of apoE, which binds to extracellular matrix via HSPG or other matrix elements, suggesting that the mechanism of the interaction may be Ca^{2+}-dependent [35]. Thus, we studied the effects of Ca^{2+} on apoE-binding to heparin. While the presence of 1 mM Ca^{2+} did not change the K_D of the interaction, metal chelators such as EDTA and DETAPAC increased dissociation of the complex. The data indicate that metal ions are not critical in the binding but that they are likely able to stabilize apoE-heparin complex. We suggested that trace bivalent cations may partially stabilize apoE-GAG complex, serving as non-specific ionic bridge(s). Interestingly, several GAGs were found to suppress Cu^{2+}-induced oxidation of VLDL apoE and the effects did not depend on their chelator activities [36]. However, possible metal-specific effects other than Ca^{2+} on apoE-GAG interaction remain to be elucidated.

Two heparin-binding sites of apoE were identified and characterized [3]. The C-terminal heparin-binding site is exposed only in lipid-free apoE. In this work, we found that apoE reconstituted with lipid exhibits affinity for heparin close to that of the free protein. Thus, it might be suggested that the high affinity binding site of apoE localized in the N-terminal domain and exposed in lipoproteins is the most important for apoE-binding to heparin.

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