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Small HDL form via apo A-I a complex with atrial natriuretic peptide

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Abstract

The goal of this study was to test the ability of small high density lipoproteins (small HDL) to bind human α-atrial natriuretic peptide (α-hANP), an amyloidogenic peptide whose involvement in cardiac pathologies is gaining increasing clinical evidence. After incubation of HDL with labeled ANP, the peptide associated to lipoprotein was detectable only in small HDL containing preparations. HDL-associated α-[^{125}I]hANP was subjected to chromatography, electrophoresis, and autoradiography. The autoradiograph showed two radioactive bands, whose molecular weight was consistent with the chromatographic pattern. Immunoblotting showed the presence of apo A-I in both autoradiographic bands. The proteins of the main band were electroeluted, incubated with labeled ANP, and subjected to two-dimensional electrophoresis followed by autoradiography. The mass spectrometry and molecular weight analyses of the radioactive spot demonstrated the presence of an apo A-I dimer. This finding provided a novel solid evidence that small HDL via apo A-I dimer are involved in the ANP sequestration and thus may play a role in preventing amyloid fibril formation.

Keywords: Amyloidosis; Atrial natriuretic peptide; HDL; Apo A-I; Two-dimensional gel electrophoresis

The amyloidoses are a group of diseases where proteins or protein fragments change from their native soluble forms into insoluble aggregates or plaques and accumulate in a variety of organs and tissues. Amyloid deposition may be either a primary process without known antecedent or secondary to some other conditions; it may be localized to one specific site or generalized throughout the body (systemic), usually with fatal consequences [1].

To date, nearly 20 different amyloidoses have been described [2–8] including cardiac amyloidosis, a relatively common finding in elderly patients which involves either the ventricles as well as the atria (senile cardiac amyloid) [9] or, more frequently, the atria alone (isolated atrial amyloid, IAA) [10]. Unlike senile cardiac amyloid which is derived from transthyretin (TTR), immunohistochemical and biochemical studies indicate that mature atrial natriuretic peptide (ANP 1–28, α-ANP) is a major immunoreactive component of IAA fibrils [11].

It is unclear why some peptide hormones, like ANP, give rise to amyloid fibrils. It is known that peptide aggregation and fibril formation are greatly influenced by various environmental factors, the presence of amino acid substitutions or post-translational modifications, as well as by peptide concentration [12–15]. Indeed, a relationship between high ANP plasma levels and the incidence and the degree of IAA has been described [16].

The clinical relevance of IAA and the amyloidogenic role of ANP have been rather undervalued but are gaining an increasing attention. In fact, recent studies [17] report that IAA progression and consequences correlate not only with aging but also with the presence of cardiac pathologies related to the synthesis and secretion of ANP thus accelerating amyloid fibril deposition.

In a previous work [18], we have demonstrated that ANP has great propensity to self-aggregation in vitro.

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In fact, incubation of a physiological amount of α-[^{125}I]hANP in PBS at 37 °C for 24 h led to the formation of a macromolecular form consisting of self-aggregated ANP monomers. The addition of plasma proteins to the incubation mixture gave rise to heterogeneous macromolecular forms, consisting of both self-aggregated and protein-bound ANP; in this case the relative amount of self-aggregated ANP was strongly reduced. Following this observation, we pointed to a protecting role of plasma-binding factors against ANP fibrillar polymerization in vivo. Moreover, we confirmed that the binding is not saturable or reversible, thus acquiring the peculiarity of a sequestration phenomenon. More recently, our findings suggested an involvement of HDL in this phenomenon [19].

Human HDL are a fraction of plasma lipoproteins which do not constitute stable entities in vivo but rather are continuously modified in the bloodstream through the action of specific factors. In their mature form, HDL consist of spherical particles with α electrophoretic mobility containing two main apo$_s$: apo A-I and apo A-II which account for 70% and 20% of the HDL proteins, respectively. Other minor apo$_s$ are present, including apo A-IV, apo$_s$ C, apo D, apo E, and apo J [20,21]. A small amount of HDL consists of discoidal particles with pre-β electrophoretic mobility containing two molecules of apo A-I complexed with phospholipids but little or no apo A-II, triglyceride, or cholesterol [22]. Although reverse cholesterol transport is probably their main function, it is current opinion that HDL likely might mediate several still poorly defined physiological functions [23,24]. A recent development in this field has just been the discovery that HDL share protective effect against the amyloidosis, by inhibiting or strongly reducing the formation of amyloid fibrils. In fact, protein constituents of HDL have been shown to bind and form complexes with Aβ and TTR [25–27].

We have recently found that HDL influence the tendency of ANP to self-aggregate since virtually fewer oligomers were detectable in co-incubations of ANP and HDL [19]. Furthermore, this study demonstrated that both spherical as well as discoidal HDL particles, medium and small HDL, respectively, associate in vitro with ANP, although displaying different binding capacities. In fact, small HDL associated with ANP more avidly than medium HDL [19].

On the other hand, no direct information regarding which HDL component interacts with ANP exist so far. Thus, having established that HDL profoundly influence ANP spontaneous aggregation, the present study was undertaken with the aim of elucidating whether the effect of HDL might be related to apo A-I, as a major, if not exclusive, component of small HDL. The results presented here provided a novel evidence that a direct interaction between ANP and small HDL via apo A-I dimer does take place. This study underlies the concept that the stabilization of soluble forms of amyloidogenic proteins by endogenous factors may be physiologically relevant and can represent a general strategy for inhibiting aggregation and amyloid formation in vivo.

Materials and methods

Materials. Equipment and all reagents of the highest available grade, unless otherwise indicated, were purchased from Amersham Biosciences.

Ligand binding of α-[^{125}I]hANP to HDL in solution. Lyophilized α-[^{125}I]hANP (specific activity 2000 Ci/mmol) was dissolved in ultrapure water according to the manufacturer's instructions. Commercial HDL preparations isolated from human plasma were supplied either from Fluka (HDL-F) (density: 1.063 to >1.21 g/ml, Fluka Sigma–Aldrich s.r.l Milano, Italy) or Calbiochem (HDL-C) (density: 1.063–1.2 g/ml, Calbiochem, Inalco S.p.A Milano, Italy). Physiological concentrations of ANP and commercial HDL were incubated at 37 °C in a water bath for 24 h, as previously reported [19]. The separation of free from bound [^{125}I]ANP (big α-[^{125}I]hANP) was achieved by adding a dextran-coated charcoal (DCC) mixture, prepared as reported in a previous work [18]; after centrifugation, the supernatant containing big α-[^{125}I]hANP was counted and expressed as percent of the total radioactivity added to incubation mixture.

Gel chromatography. Commercial HDL or big α-[^{125}I]hANP samples were submitted to gel filtration chromatography using Sephacryl S 300 HR column (1.6 × 60 cm). The details are reported in a previous work [19]. In synthesis, the column was equilibrated at room temperature in elution buffer containing 0.15 M NaCl, 0.05 M Na-phosphate, NaN_3 (0.02%, w/v), and 1 mM EDTA, pH 7.0, and eluted with flow rate of 30 ml/h; loading volumes were 1 ml. Fractions (1 ml) were collected, monitored for protein content by measurement of A_{280} or counted for ^{125}I. The recovery of radioactivity ranged from 80% to 90% of the total applied. The apparent molecular size of the labeled material in the fractions was determined by comparing its elution volume (V_e) with that of commercially available gel filtration protein calibration mixtures.

SDS–PAGE and autoradiography. Big α-[^{125}I]hANP, treated or untreated with 14.4 mM of 2β-mercaptoethanol, was subjected to 12.5% SDS–PAGE, using a Mini-Protean II Cell electrophoresis system (Bio-Rad, Milano, Italy). Gels were stained for protein with 0.1% Coomassie Blue R-250 in water/methanol/glacial acetic acid (45:45:10, by vol.) and destained in water/methanol/glacial acetic acid (80:10:10, by vol.) solution. Molecular weight standards (14–94 kDa) were parallely run. Then, gels were dried and exposed overnight at −70 °C to Kodak X-Omat AR autoradiography film and a single intensifying screen. Amount of total proteins loaded per lane was 50 μg.

Immunoblot analysis. Samples were transferred from gels to nitrocellulose in Mini Trans-Blot Transfer Cell (Bio-Rad, Milano, Italy); the buffer was 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol. The electroblots were bathed for 1 h in PBS containing 5% (w:v) nonfat dried milk (blocking buffer) and, after washing in PBS, 0.05% (v/v) Triton X-100, for 1.5 h in blocking buffer plus 0.05% Triton X-100 (incubation buffer) containing polyclonal antibody against apo A-I (Calbiochem, Inalco S.p.A. Milano, Italy) at 1:10,000 dilution. The blots were washed as above and then bathed for 1.5 h at 37 °C in the incubation buffer containing peroxidase conjugate goat anti-rabbit IgG (Bio-Rad, Milano, Italy) used at a 1:3000 dilution. After washing of the membrane, immunolabeled proteins were visualized by enhanced chemiluminescence (ECL).

Ligand binding of α-[^{125}I]hANP to electroeluted protein from SDS–PAGE. Following SDS–PAGE, gels were rinsed with double-distilled H_2O, and a strip of the gel, including standards and sample, was cut with a razor for staining with Coomassie Blue R-250. Not stained gel slices, containing the 45–67 kDa region, were excised for electroelution.

Electroelution was performed by using Electro-Eluter (Bio-Rad, Milano, Italy) and the eluting buffer consisted of 50 mM NH_4HCO_3, 0.1% (w/v) SDS. The eluted proteins were lyophilized, dissolved in PBS, and incubated with α-[^{125}I]hANP at 37 °C in a water bath for 24 h. Big α-[^{125}I]hANP was denatured in sample buffer for two-dimensional electrophoresis (2D-PAGE).

2D-PAGE and autoradiography. Commercial preparation of HDL and big α-[^{125}I]hANP was denatured in the buffer for the first dimension of 2D-PAGE consisting of a solution containing 8 M urea, 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 40 mM Tris, 65 mM dithioerythritol (DTE), and a trace of bromophenol blue. 2D electrophoresis was carried out according to procedures detailed elsewhere [28]. Forty-five micrograms of protein sample was applied to an Immobiline strip (IPG, Immobilized pH Gradient) consisting of a non-linear gradient, pH range 3.5–10, previously rehydrated. Isoelectric focusing was carried out on Multiphor II. The voltage was linearly increased from 300 to 3500 V during the first 3 h and then stabilized at 5000 V for 22 h (total 110 kV h). The IPG strip was then equilibrated in 6 M urea, 30% (w/v) glycerol, 2% (w/v) SDS, 0.05 M Tris–HCl, pH 6.8, 2% (w/v) DTE, and then with 2.5% (w/v) iodoacetamide. Electrophoresis in the second dimension was carried out on a 9–16% polyacrylamide non-linear gradient gel ($18 \times 20\,\text{cm} \times 1.5\,\text{mm}$) at a constant current of 40 mA. DTE was omitted in all steps when big α-[^{125}I]hANP was analyzed. The gels were stained with silver nitrate as previously described [28], dried, and exposed for 3 weeks at −70 °C to Kodak X-Omat AR autoradiography film and a single intensifying screen. The digitalized images were obtained by Laser Densitometer (Molecular Dynamics, Sunnyvale, CA, USA) scanning of the gels and then analyzed qualitatively and quantitatively by the Melanie II 2D-PAGE or PD-Quest software (Bio-Rad, Hercules, CA).

Mass spectrometry. Radioactive spot from 2D-PAGE was excised from the gel, triturated, and washed with water and acetonitrile. Proteins were in-gel reduced, *S*-alkylated, and digested with trypsin as previously reported [29]. Peptide mixture was loaded on the MALDI target, using the dried droplet technique and α-cyano-4-hydroxycinnamic as matrix, and analyzed by using Voyager DE-PRO mass spectrometer (Applied Biosystems, Framingham, MA, USA). Internal mass calibration was performed with peptides deriving from trypsin autoproteolysis. PROWL software package was used to identify spots unambiguously from independent non-redundant sequence database [29].

Results and discussion

To identify the HDL components interacting with ANP, we used two commercial HDL preparations (Fluka and Calbiochem, HDL-F and HDL-C, respectively) obtained from human plasma by sequential ultracentrifugation. Both types of HDL were analyzed for their protein components by 2D-PAGE. This biochemical characterization was necessary to evaluate the protein repertoire of HDL preparations which, actually, differed significantly in their protein content. As reported in Fig. 1, HDL-C (B) contained almost exclusively Apo_s, while in HDL-F (A) some additional plasma proteins were present.

Both HDL were then incubated with α-[^{125}I]hANP for 24 h at 37 °C. The production of big α-[^{125}I]hANP was observed only when HDL-F were utilized. To determine which HDL subfraction was associated with ANP, we utilized gel filtration chromatography on Sephacryl S-300 HR column either for HDL-F fractionation or for radioactivity distribution among subfractions. As shown in Fig. 2 (Profile A), the chromatographic procedure yielded three peaks which eluted either at the void volume (Vo) or before albumin and after ferritin or slightly after albumin. Analogous protein distribution was obtained with several different lots of commercial HDL-F. In a previous work [19], we had identified the initial small peak as large HDL, the major peak as medium HDL, and the last peak as small HDL. As the chromatographic profiles were completely superimposable, in the present work we adopted the same nomenclature. Fig. 2 (Profile B) showed that no radioactivity was detectable in the Vo, where self-aggregated ANP monomers elute, as previously reported [19]. Except for a very small peak in the area of the medium HDL, all big α-[^{125}I]hANP eluted from the gel filtration column in only one peak, corresponding to the small HDL peak (Profile A).

The radioactive ANP–HDL protein complexes were resolved by SDS–PAGE followed by autoradiography. As shown in Fig. 3 (lane 1), three radioactive bands were apparent: a >94 kDa band, migrating near the origin of the resolving gel, an evident 45–67 kDa band, and a faint band in the dye front. These results agreed reasonably

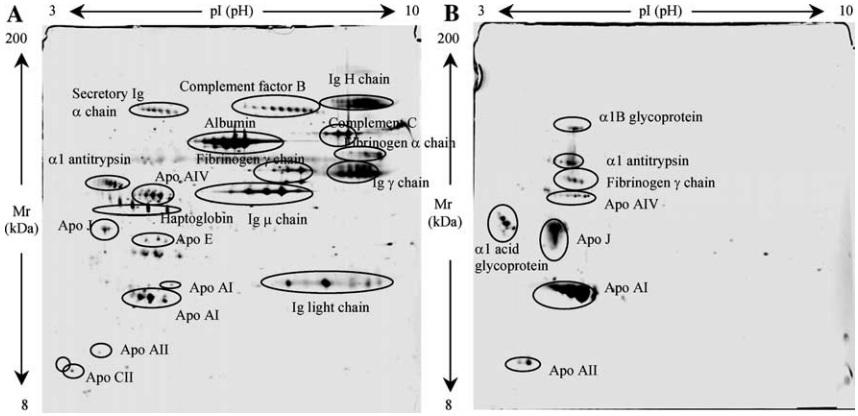

Fig. 1. 2D-PAGE analysis of protein components of HDL-F (A) and HDL-C (B). Plasma proteins were identified by gel matching with the human plasma 2D-gel stored in the Swiss-2DPAGE database.

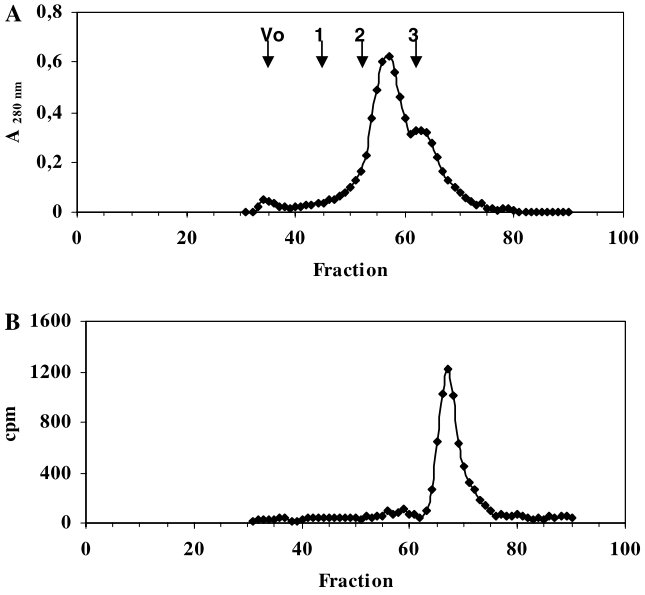

Fig. 2. Representative gel filtration profiles on Sephacryl S-300 HR of commercial HDL-F (Profile A) and big α-[^{125}I]hANP generated upon incubation at 37 °C for 24 h with HDL-F (Profile B). Arrows with numbers designate elution fractions for the following molecular weight markers: 1, thyroglobulin (669 kDa); 2, ferritin (440 kDa); and 3, albumin (67 kDa). Vo, void volume. Similar profiles were obtained in a second experiment.

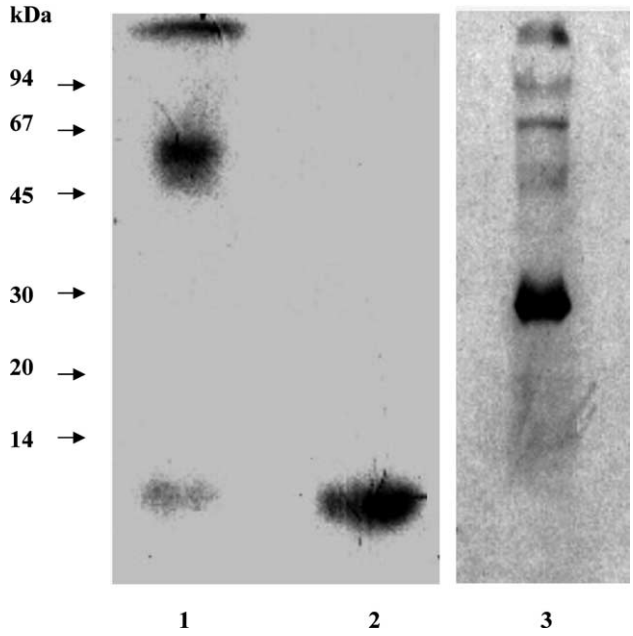

Fig. 3. Autoradiographical and immunological patterns of big α-[^{125}I]hANP. After incubation of α-[^{125}I]hANP and HDL-F, big α-[^{125}I]hANP was applied on 12.5% SDS–PAGE in non-reducing and reducing conditions followed by autoradiography (lane 1 and lane 2, respectively); immunoblotting was performed on non-reduced sample with the anti-apo A-I polyclonal antibody (lane 3).

well with findings obtained when big α-[^{125}I]hANP was separated by gel filtration chromatography (Fig. 2, Profile B). In fact, the radioactive bands of apparent molecular weight on SDS gel of >94 and 45–67 kDa may represent radiolabeled proteins which eluted on a Sephacryl SH-300 column in the medium size HDL range and in the small size HDL region, respectively. In accordance with our previous findings on big α-[^{125}I]hANP stability [18], HDL were found to form SDS-stable complexes with ANP. These forms dissociated in the presence of a reducing agent with a corresponding increase of free monomeric α-[^{125}I]hANP (Fig. 3, lane 2). The stability feature of ANP-complexes was similar to that reported for Aβ [26,30]. The sample subjected to SDS–PAGE under non-reducing conditions was analyzed for apo A-I by immunoblotting. As shown in Fig. 3 (lane 3), the radioactive bands with molecular weight greater than 45 kDa were immunostained. Thus, immunoblotting revealed the presence of monomeric apo A-I (28 kDa) and some SDS-stable protein complexes, all containing apo A-I.

In order to investigate protein species other than apo A-I occurring in these complexes, the proteins electroeluted from the SDS-gel region ranging between 45 and 67 kDa were incubated with α-[^{125}I]hANP and submitted to 2D-PAGE, followed by autoradiography. As shown in Fig. 4, several protein components were present in the one-dimensional PAGE band which could be resolved by 2D-PAGE. Surprisingly, autoradiography revealed the presence of an ANP-binding protein in a gel region with a low protein content (Fig. 4). Nevertheless, mass spectrometry and molecular weight analyses unambiguously identified the ANP-binding protein as a human apo A-I dimer.

Our previous studies [18,19] have demonstrated that circulating ANP, in analogy with other amyloidogenic peptides, is sequestered by human plasma components, which play an important role in regulating ANP aggregation and fibrillar polymerization. In fact, since the formation of amyloid fibrils generally takes place by a nucleated growth process [31] that is dependent on local peptide concentration, the ANP sequester phenomenon could be of importance in delaying and/or inhibiting the formation of an amyloid nidus. Although we could not have been able to identify the precise endogenous factor affecting the rate of amyloid formation, the interaction of a complex between commercial HDL and ANP had been clearly demonstrated [19]. These observations were in line with reports describing an association of Aβ with HDL apolipoproteins in body fluids of normal individuals [25,26,32]. Since the major apolipoproteins of HDL are apo A-I and apo A-II, in preliminary experiments we tested these isolated apo$_s$ for their ability to bind labeled ANP in solution. Although no direct binding was observed (not shown), our results did not exclude the possibility that the physiologic integrity of HDL particles could be required for ANP–HDL association, as already suggested for Aβ [26]. In addition, our data [19]

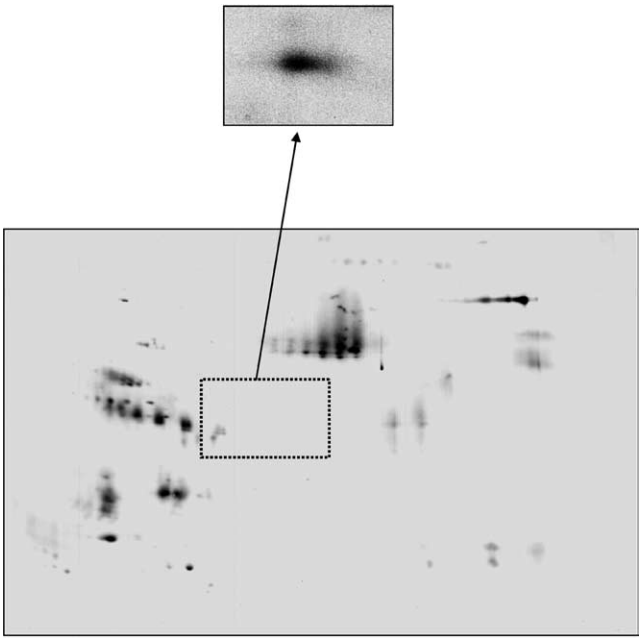

Fig. 4. Protein repertoire from the SDS–PAGE electroeluted region (45–67 kDa). Two-dimensional electrophoresis of electroeluted proteins was performed as described in Materials and methods. The 2D-gel was run under non-reducing conditions. The insert shows the part of the gel where bound ANP was revealed by autoradiography.

strongly suggested that the small HDL particles were the major players in the ANP-binding phenomenon.

On account of all these observations, for the present study α-[^{125}I]hANP free of pre-formed aggregated was chosen together with a commercial preparation of HDL, which contained also small HDL particles. With regard to the first choice, we knew [18] that ANP aggregates contaminating commercial preparations of the peptide greatly influence the process of fibril formation by acting as a seed for further polymerization and thus masking the binding process.

With regard to the second choice, it is well known that small apo A-I containing particles with pre-β electrophoretic mobility [22] are recovered only in the $d > 1.21$ g/ml fraction [33], following the plasma sequential ultracentrifugation. Consequently, these particles were present in the commercial preparation supplied from Fluka and absent in that supplied from Calbiochem.

In experiments where HDL-F and radioiodinated ANP were incubated and then subjected to the gel filtration chromatography, big α-[^{125}I]hANP was recovered in fractions roughly corresponding to those of small HDL. These results agreed with our previous observations [19] and further supported the involvement of this lipoprotein fraction, consisting almost exclusively of apo A-I [22], in the ANP binding. SDS–PAGE analysis of big α-[^{125}I]hANP, followed by autoradiography and immunoblotting, revealed a quantitatively more prominent radioactive band of 45–67 kDa, immunostained by anti-apo A-I antibody. To further investigate the apo A-I role, ligand-binding experiments were performed by incubation of proteins electroeluted from this gel region with α-[^{125}I]hANP, followed by 2D-PAGE and autoradiography. The mass spectrometry analysis revealed that apo A-I was the unique protein present in the only a radioactive spot evidentiated in the gel area corresponding to a region roughly covering the p*I* 5.00–5.80 and molecular weight 35–55 kDa ranges.

Given the molecular weight of the spot, we assumed that the association of ANP with small HDL occurred via an apo A-I dimer. In fact, it is known that the molecular mass of small HDL containing two copies of apo A-I ranges between 40 and 60 kDa [22]. The preferential association of ANP with small HDL was further strengthened by the lack of ANP complexed when radiolabeled peptide was incubated with HDL-C free of small HDL. As reported for apo A-I–Aβ and apo E–Aβ interactions and previously suspected by us [19], it is likely that the combination of the poor lipid status of apo A-I containing particles [34,35] and the amphiphilic nature of ANP [36] is responsible for the selective small HDL affinity.

Nevertheless, we do not exclude the possibility that part of ANP is sequestered by plasma proteins other than apo AI-HDL. In fact, our unpublished in vitro findings indicate that plasma contains at least four times amyloid inhibitory activity than HDL. Since it has been recently reported that the ability to form amyloid fibrils is not only a property of the amyloidogenic peptides identified to date but is a generic feature of polypeptide chains [37,38], it is likely that nature has provided the organism with a panel of different binding proteins to inhibit fibril formation. The existence of specific inhibitory factors for families of amyloidogenic peptides could represent a strategy to avoid the saturation of the system. On this subject further detailed studies are needed to identify new factors which bind ANP. A better knowledge of these factors and the mechanisms regulating their activity may be of great importance in therapeutic approaches for the prevention of diseases caused by amyloidosis.

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