A Synthetic Peptide That Inhibits Lipoprotein(a) Assembly

Rebecca J. Sharp, Matthew A. Perugini, Santica M. Marcovina, Sally P.A. McCormick

Objective—We previously reported that human apolipoprotein B100 (apoB) amino acids 4330–4397 were important for the initial noncovalent binding to apolipoprotein(a) [apo(a)] that facilitates lipoprotein(a) [Lp(a)] assembly. In this study, we aimed to further define the apoB sequences within the 4330–4397 region that were important for the noncovalent binding to apo(a).

Methods and Results—Alignment of the human apoB4330–4397 sequence with mouse apoB, which also noncovalently binds apo(a), revealed stretches of similar sequence, including a lysine-rich sequence spanning apoB amino acids 4372–4392. Structural analysis of the apoB4372–4392 sequence using the WHEEL program predicted an amphipathic α-helix. Circular dichroism studies of a synthetic peptide spanning human apoB amino acids 4372–4392, both in the absence and presence of dimyristoylphosphatidylcholine, confirmed the α-helical nature of the sequence. We tested the ability of the apoB4372–4392 peptide to bind to apo(a) and found that the peptide bound to apo(a) with high affinity but not to Lp(a). The apoB4372–4392 peptide inhibited Lp(a) assembly in Lp(a) formation assays far more effectively than the lysine analogue, ε-amino-n-caproic acid (IC₅₀=40 μmol/L versus 10 mmol/L, respectively). Incorporation of the apoB4372–4392 peptide onto dimyristoylphosphatidylcholine vesicles yielded an even more effective inhibitor (IC₅₀=4 μmol/L).

Conclusions—Our study shows that the apoB4372–4392 sequence mediates the initial noncovalent binding to apo(a) and has demonstrated that the apoB4372–4392 peptide is a novel and effective inhibitor of Lp(a) assembly. (Arterioscler Thromb Vasc Biol. 2003;23:502-507.)

Key Words: lipoprotein(a) ■ assembly ■ apolipoprotein B ■ apolipoprotein(a) ■ peptide inhibitor

Lipoprotein(a) [Lp(a)] is an atherogenic lipoprotein formed by the disulphide linkage of apolipoprotein(a) [apo(a)] to apolipoprotein B100 (apoB) on a low-density lipoprotein (LDL).¹ Mutagenesis studies have identified apo(a)Cys4057² and apoBCys4326⁴ as the two cysteine residues involved in the disulphide link. A mutant apoB lacking Cys4326 still binds apo(a) noncovalently despite being unable to form the disulphide bond.⁶ This observation supports the two-step model of Lp(a) assembly in which apo(a) and apoB bind together noncovalently to subsequently facilitate the formation of the disulphide bond. The apoB sequences required for the initial noncovalent interaction with apo(a) have not been fully characterized; however, several studies have implicated the involvement of apoB lysine residues. Lysine analogues disrupt Lp(a) assembly in vitro,⁸–¹⁰ and deletion of apo(a) kringle IV domains containing lysine-binding sites interferes with Lp(a) formation.¹¹–¹³

Over the past few years, we have focused our studies on identifying the human apoB sequences that are required for the initial noncovalent binding to apo(a). The carboxyl-terminal sequences of apoB were found to be important for the interaction with apo(a) in a study of 2 truncated apoB proteins, apoB95 (4330 amino acids) and apoB97 (4397 amino acids).¹⁴ In that study, the apoB97 protein formed Lp(a) with an efficiency similar to full-length apoB100, whereas apoB95 was severely impaired in its ability to bind apo(a). This suggested that the region between apoB amino acids 4330 and 4397 was important for the noncovalent interaction of apoB with apo(a).

Other species of apoB, besides human, can interact with apo(a).¹⁵ Mouse apoB is of interest because it is well documented that mouse apoB can associate noncovalently with apo(a) despite lacking the Cys4326 residue.⁶,¹⁶,¹⁷ The region spanning apoB amino acids 4330–4397 is well conserved between human and mouse and is located in a region predicted to contain several amphipathic α-helices.¹⁸ Interestingly, the 4330–4397 region contains a cluster of 4 lysine residues in a 21–amino acid sequence, spanning human apoB amino acids 4372–4392. These observations have led us to hypothesize that one or more of the lysine residues in the apoB4372–4392 sequence may play an important role in the first step of Lp(a) assembly.

To test the importance of the apoB4372–4392 sequence for the noncovalent interaction of apoB with apo(a), a synthetic
peptide spanning human apoB amino acids 4372–4392 was characterized structurally and evaluated for its ability to noncovalently bind apo(a) and Lp(a). The peptide was tested for its ability to inhibit Lp(a) assembly both in the absence and presence of phospholipid.

Methods

Materials

Two synthetic apoB peptides, apoB4372-4392 (KYYELEEKIVSLINKKLWALVK) and a control peptide, apoB4372-4392scram (VKEYKYSKLKLNLEAYEYLL), were synthesized by Chiron Mimotopes using an Applied Biosystems model 430A synthesiser. Both peptides were purified by high-performance liquid chromatography and found to be >90% pure by mass spectrometry analysis. Human apo(a) transgenic mice were obtained from Dr Robert Hammer (University of Texas Southwestern Medical Center, Dallas, Texas) and human apoB transgenic mice from Dr Stephen Young (Gladstone Institute of Cardiovascular Disease, San Francisco, Calif). Human Lp(a) transgenic mice were generated as previously described. All mice were housed in a conventional facility at the University of Ottawa and maintained on a regular chow diet. Wild-type mouse plasma was obtained from nontransgenic littersmates. The MAb a-6 and horse-radish peroxidase (hrp)-labeled MAb a-5 monoclonal antibodies specific for human apo(a) have been previously described. The human apoB-specific monoclonal antibody, 1D1, was a gift from Dr Ross Milne (University of Ottawa Heart Institute, Ottawa, Ontario). Dimyristoylphosphatidylcholine (DMPC) was purchased from Sigma-Aldrich, and an enzymatic phospholipid assay kit was supplied by Roche Diagnostics. Superdex Peptide HR 10/30 columns were purchased from Amersham Biosciences, and microtitre plates (Costar) were purchased from Corning. PBS tablets were purchased from Oxoid. BSA was obtained from Boehringer Mannheim and casein hydrolysate from Gibco BRL. The enhanced chemiluminescence detection kit was from Amersham Pharmacia Biotech. All other chemicals were purchased from Sigma-Aldrich, BDH Chemicals, or Roche Molecular Biochemicals.

Preparation of Phospholipid Vesicles

Small unilamellar vesicles of DMPC were prepared using a method similar to that described by New. Briefly, DMPC suspended in 5 mmol/L sodium phosphate, 100 mmol/L NaCl, pH 7.0, was sonicated using a 9.5-mm probe sonicator (Soniprep 150, MSE Scientific Instruments) at 20 MHz for 10 seconds with 30-second breaks in between. The temperature was maintained at 30°C using a jacketed vial with a circulating water bath above the phase transition temperature of DMPC (24°C). The vesicles were centrifuged at 2500g for 5 minutes to pellet titanium originating from the sonicator probe. Phospholipid concentrations were determined at 20°C using an enzymatic phospholipid assay kit.

Circular Dichroism Measurements

Before circular dichroism (CD) measurements, the apoB4372-4392 peptide was dissolved in 5 mmol/L sodium phosphate and 100 mmol/L NaCl, pH 7.0, and purified on a Superdex Peptide HR 10/30 prepacked column using a flow rate of 1 mL/min. Spectra of pure peptide samples in the absence and presence of DMPC vesicles were recorded at 25°C on an Aviv 62DS CD spectrophotometer using a 1-mm pathlength quartz cuvette, recording between the wavelengths of 195–250 nm, with a step size of 0.5 nm and a slit bandwidth of 1.5 nm. Signal averaging time was 1 second, and ellipticities were reported as mean residue ellipticity (θ) in deg cm² dmol⁻¹. The concentration of apoB4372-4392 peptide used in the CD studies (120 μg/mL) was determined by absorption spectroscopy on a Cary 5 UV/Vis spectrophotometer at 280 nm (ε280 = 2980). Freshly prepared DMPC vesicles were added to a 120-μg/mL (47-μmol/L) sample of apoB4372-4392 up to a final concentration of 4.4 mmol/mL DMPC (DMPC:peptide molar ratio of 105:1). The change in mean residue ellipticity at 222 nm (θ222 nm) with increasing DMPC was recorded to determine the DMPC:peptide molar ratio at which the peptide was saturated with phospholipid. The α-helical content of the apoB4372-4392 peptide in the absence and presence of a saturating amount of DMPC was calculated from θ222 nm according to Aggerbeck et al. as follows: %α-helix = (θ222 nm − 2340)/303.

Ap(o)a and Lp(a) Binding Studies

The ability of the apoB4372-4392 and apoB4372-4392scram peptides to bind apo(a) and Lp(a) was measured using a modification of an enzyme-linked ligand sorbent assay (ELLSA) reported by Herrmann et al. A Costar polystyrene microtitre plate was coated with 5 μg per well of peptide in 0.1 mol/L carbonate buffer and 2 mmol/L MgCl₂, pH 9.6, for 15 hours at 25°C. Peptide solution was evaporated by drying at 37°C for 48 hours, and the plates were washed 3 times with wash solution (10 mmol/L Tris, 0.15 mol/L NaCl, pH 7.45) to remove excess peptide. Apo(a) transgenic mouse plasma, Lp(a) transgenic mouse plasma, and wild-type mouse plasma (negative control) were diluted 1:10 with diluent (0.1 mol/L phosphate, 1 mol/L NaCl, 7.5 mmol/L KCl, 0.3% casein hydrolysate, 0.005% thimerosal, and 0.01% Tween 20, pH 7.45). Additional dilutions of the plasma samples were performed with a 1:10 dilution of wild-type mouse plasma to standardize the amount of mouse plasma added to each well. The diluted plasma samples (50 μL) were incubated on the peptide-coated plates for 90 minutes at 28°C to allow binding of apo(a) and Lp(a) to the peptides. Plates were washed 3 times with wash solution. Bound apo(a) and Lp(a) were detected by incubating with a 1:1000 dilution of the hrp-labeled MAb a-5 antibody in a 1:10 dilution of diluent. Plates were washed 3 times with wash solution and then developed with 100 μL of substrate solution (0-phenylene-diamine dihydrochloride and hydrogen peroxide). Reactions were stopped by addition of 100 μL of 2 mol/L H₂SO₄ and the absorbance at 490 nm read on a BIO-TEK EL340 plate reader. The level of background binding was determined from the wild-type plasma sample and this was subtracted from both the apo(a) and Lp(a) binding curves.

In Vitro Lp(a) Formation Assays

The ability of the apoB4372-4392 peptide to inhibit the noncovalent interaction between apo(a) and apoB and prevent disulfide bond formation was assessed using an in vitro Lp(a) formation assay. Plasma (1 μL) from an apo(a) transgenic mouse was incubated with plasma (2 μL) from a human apoB transgenic mouse for 3 hours at 37°C in the presence of increasing amounts (1 to 400 μmol/L) of the apoB4372-4392 peptide either in the absence or presence of a 45:1 molar ratio of DMPC:peptide. Similar incubations containing apo(a), human apoB, and increasing amounts of either the apoB4372-4392scram control peptide or the lysine analogue ε-amino-n-caproic acid (ε-ACA) were also performed. Samples were size-fractionated on SDS/4% polyacrylamide gels under nonreducing conditions, and the separated proteins were transferred to nitrocellulose for Western blotting with the hrp-labeled MAb a-5 antibody. Lp(a) and apo(a) bands were visualized using the enhanced chemiluminescence detection system. The amounts of Lp(a) formed in the incubations containing the apoB4372-4392 peptide and ε-ACA were also quantified by a Lp(a)-specific enzyme-linked immunosorbent assay (ELISA).

Lp(a) ELISA

The relative amounts of Lp(a) formed in the in vitro Lp(a) formation assays were measured using a modification of the Lp(a) sandwich ELISA reported by Marcovina et al. Each well of a Costar polystyrene microtitre plate was coated with 100 μL of 100 mmol/L NaHCO₃, pH 9.6, containing 3 μg/mL of the MAb a-6 antibody for 16 hours at 4°C. Unbound antibody was removed by washing the plate 3 times with PBS, pH 7.4. Nonspecific binding sites were blocked by incubating with 300 μL of PBS containing 3% BSA at 28°C for 1 hour, and the plates were washed with PBS. Samples to be analyzed were appropriately diluted in filter-sterilized dilution buffer (PBS containing 0.1% BSA and 0.05% Tween 20, pH 7.4). Diluted samples (100 μL) were incubated on the plates in quadrup
Figure 1. Alignment of partial protein sequences from human and mouse apoB in the carboxyl-terminal region. A partial protein sequence spanning mouse apoB amino acids 4324–4403 (provided by Dr Stephen Young) was aligned with the human apoB sequence in FASTA format using the LALIGN program on the ExPASy Molecular Biology Server (http://www.expasy.ch).

Previous structural analysis of apoB using the computer program LOCATE18 predicted that the 4372–4392 sequence lies within a class A amphipathic α-helix with high lipid affinity. A helical wheel diagram of the 4372–4392 sequence was produced using the WHEEL program29 (Figure I, available online at http://atvb.ahajournals.org). Within the helix, the side chains of the 4 lysine residues are found at the lipid/aqueous interface projecting into the aqueous phase, whereas the side chains of the hydrophobic residues (in boldface) are predicted to be buried in the lipid phase. The predicted α-helical structure shown in Figure I was confirmed by CD spectroscopy studies of a synthetic peptide spanning apoB residues 4372–4392 (apoB4372–4392) in the absence and presence of DMPC vesicles (Figure 2). The CD spectrum of apoB4372–4392 in the absence of DMPC (●) is typical of an α-helical peptide, showing characteristic double minima at 208 and 222 nm, respectively. Titration of the apoB4372–4392 peptide with increasing amounts of DMPC demonstrated that there was significant binding of the peptide to the phospholipid vesicles. This is shown by the increase in mean residue ellipticity at 222 nm as a function of increasing DMPC:peptide molar ratio (Figure 2, inset). Saturation of the peptide with DMPC was achieved at a DMPC to peptide molar ratio of ~45:1, which corresponds to ~2.1 phospholipid molecules per amino acid. This ratio was used for all subsequent experiments involving the apoB4372–4392 peptide in the presence of DMPC. The CD spectrum of the apoB4372–4392 peptide complexed to DMPC vesicles (Figure 2, ○) shows an increase in α-helicity compared with the lipid-free peptide, indicating that the binding of the peptide to phospholipid stabilizes the α-helical structure. According to the Aggerbeck equation, the α-helical content of the peptide in the absence and presence of saturating amounts of DMPC is ~68% and 88%, respectively.

To test whether the apoB4372–4392 sequence plays a role in Lp(a) assembly, we assessed the ability of the apoB4372–4392 peptide to bind to apo(a). Apo(a) binding studies showed that the immobilized apoB4372–4392 peptide bound avidly to apo(a) in transgenic mouse plasma (Figure 3), whereas the apoB4372–4392scram control peptide shows no apparent binding to apo(a). In contrast, the binding of apoB4372–4392 to Lp(a) in transgenic mouse plasma was minimal (Figure II, available online at http://atvb.ahajournals.org), as was the binding of the apoB4372–4392scram peptide.

We then tested whether the apoB4372–4392 peptide could inhibit Lp(a) assembly in vitro. Increasing amounts of the peptide were incubated with fixed amounts of apo(a) and human apoB in a Lp(a) formation assay. Western blot analysis of incubations containing increasing amounts of the apoB4372–4392 peptide showed a reduction in Lp(a) formation and a corresponding increase in free apo(a), indicative of a
similar concentrations of the apoB 4372 peptide to apo(a) was measured with a solid-phase ELLSA assay. The peptides were immobilized on microtitre plates and incubated with increasing amounts of apo(a) contained in diluted apo(a) transgenic mouse plasma. Bound apo(a) was detected with the MAb a-5 antibody. Data points represent the average of the absorbance values obtained from quadruplicate incubations. Error bars indicate SEM.

To investigate the inhibitory capacity of the apoB 4372-4392 peptide further, an Lp(a) ELISA assay was used to determine an IC\textsubscript{50} value for the peptide both in the absence and presence of DMPC (Figure 5). For comparison, an IC\textsubscript{50} value was also obtained for the lysine analogue \textepsilon-ACA. The apoB 4372-4392 peptide proved to be an effective inhibitor of Lp(a) formation with an IC\textsubscript{50} of 40 \textmu mol/L. The DMPC-complexed peptide was an even better inhibitor, with an IC\textsubscript{50} value of 4 \textmu mol/L. The DMPC-complexed apoB 4372-4392 peptide was 2500-fold more effective at inhibiting Lp(a) formation than e-ACA, which showed an IC\textsubscript{50} of 50 mmol/L. The IC\textsubscript{50} value we obtained for e-ACA was comparable with values previously reported for lysine analogues using a similar \textepsilon(Apob)-formation assay to the one used here.\textsuperscript{30}

**Discussion**

High plasma levels of Lp(a) have been identified as a risk factor for developing heart disease in large clinical trials.\textsuperscript{31–33}

![Figure 4. Inhibition of Lp(a) formation by the apoB 4372-4392 peptide. Increasing amounts of the apoB 4372-4392 peptide were added to incubations containing human apo(a) transgenic mouse plasma and human apoB transgenic mouse plasma. The amount of Lp(a) formed in each incubation was assessed by separation of the incubation mix on SDS/4% polyacrylamide gels under nonreducing conditions and Western blot analysis with the MAb a-5 antibody.](https://example.com)

However, there is presently no safe and effective therapy to lower Lp(a) levels. Research aimed at identifying the protein sequences required for Lp(a) assembly may provide new targets for the design of Lp(a)-lowering agents. We previously demonstrated that apoBCys4326 was the site of covalent attachment to apo(a).\textsuperscript{4} However, the two-step model of Lp(a) assembly depicts a noncovalent association between apoB and apo(a) that precedes the formation of the disulphide bond.\textsuperscript{3,7} The exact amino acids required for the noncovalent association between apo(a) and apoB are unknown, although a growing body of evidence suggests that lysine residues in apoB may play an important role.\textsuperscript{8–13}

Studies of truncated apoB molecules have implicated the carboxyl-terminus of apoB, in particular amino acids 4330–4397, as being important for the noncovalent association with apo(a).\textsuperscript{14} We compared the human apoB4330–4397 sequence with the equivalent mouse apoB sequence, because it is well established that mouse apoB can noncovalently bind apo(a). Significant sequence identity between human and mouse apoB was evident, including a lysine-rich region of human apoB spanning amino acids 4372–4392 (Figure 1). In this study, a peptide spanning residues 4372–4392 of human apoB was synthesized and its ability to interact with apo(a) was investigated.

Apo(a) binding studies showed that the apoB 4372-4392 peptide bound to apo(a) with high affinity (Figure 3). Addition of the apoB 4372-4392 peptide to Lp(a) formation assays showed the peptide to be an effective inhibitor of Lp(a) assembly with an estimated IC\textsubscript{50} of 40 \textmu mol/L (Figure 5). Complexing of the peptide to DMPC enhanced its inhibitory capacity even further, yielding an IC\textsubscript{50} of 4 \textmu mol/L. Interestingly, the apoB 4372-4392 peptide complexed to DMPC was 2500-fold more effective at inhibiting Lp(a) formation than the lysine analogue \textepsilon-ACA, a well-known inhibitor of Lp(a) assembly.\textsuperscript{10} We propose that the apoB 4372-4392 peptide is inhibiting Lp(a) formation by competing with the apoB molecule for noncovalent binding to apo(a) (Figure 6). Once bound to apo(a), the peptide effectively blocks the binding of apo(a) to apoB, resulting in disruption to Lp(a) assembly (Figure 4). Complete inhibition of Lp(a) assembly was seen at 100 \textmu mol/L peptide. Complexing of the apoB 4372-4392 peptide to DMPC enhanced its inhibitory capacity, with complete inhibition of Lp(a) assembly being achieved at 40 \textmu mol/L peptide (Figure III, available online at http://atvb.ahajournals.org). Incubations containing similar concentrations of the apoB 4372-4392 peptide showed no effect on Lp(a) formation (Figure III, available online at http://atvb.ahajournals.org).

![Figure 5. Comparing the inhibitory capacity of the apoB 4372-4392 peptide to \textepsilon-ACA. Increasing amounts of the apoB 4372-4392 peptide (in the absence or presence of DMPC) or \textepsilon-ACA were added to incubations containing human apo(a) transgenic mouse plasma and human apoB transgenic mouse plasma. The amount of Lp(a) formed in each incubation was quantified using a Lp(a)-specific sandwich ELISA. Each incubation was measured in quadruplicate, and the average value was expressed as a percentage of Lp(a) formed relative to the incubation containing no inhibitor. Error bars indicate standard deviation from the mean. The IC\textsubscript{50} values for all 3 inhibitors are shown.](https://example.com)
The apo(a) protein has multiple copies of the plasminogen kringle 4–like domain, of which there are 10 distinct subtypes designated KIV1 through KIV10. Some of the KIV subtypes, in particular KIV6 through 8, contain weak lysine binding domains that have been shown to be important for efficient Lp(a) assembly.11–13,36 Whether all 3 kringle subtypes are required for the initial binding to apoB or whether only a single kringle subtype is necessary for Lp(a) assembly has not been fully established. The structures of apo(a) kringle IV6 and IV7 have recently been solved.37,38 In both, the lysine-binding pocket consists of an anionic center formed by Asp55 and Glu57 followed by a hydrophobic trough involving the Trp62, Tyr64, Trp72, and Tyr74 residues. Lysine analogues interact with the pocket via the amino group of their side chain that makes an ion-pair interaction with the Asp55 and Glu57 residues. A similar interaction would be expected with one of the lysine side chains in the apoB4372–4392 sequence. In addition to this interaction, there is clearly much potential for the interaction of hydrophobic side chains from the apoB4372–4392 sequence with the hydrophobic trough lining the apo(a) lysine binding pocket. Interestingly, two highly conserved tyrosine residues follow the Lys4372 residue. Additional research is needed to elucidate the exact nature of the binding of the apoB4372–4392 peptide to apo(a). Binding of the apoB4372–4392 peptide onto apo(a) kringle IV structures, combined with mutagenesis studies of the 4372–4392 sequence, will no doubt establish the exact amino acids in the 4372–4392 sequence that interact with apo(a).

Two other apoB peptides, apoB3732–3749 and apoB304–3317, have been shown to bind apo(a) with high affinity, although neither has been reported to inhibit Lp(a) assembly. A recent study by Becker et al41 showed that a synthetic apoB peptide spanning the apoB680–704 sequence has a high affinity for apo(a) (K_D = 83.4 nmol/L). Surprisingly, however, the apoB680–704 peptide only had a modest inhibitory effect on Lp(a) assembly. The inhibition data on the apoB680–704 peptide, as determined by Western blot analysis, showed the IC_50 to be ≈1 nmol/L, which is similar to the IC_50 values reported for various lysine analogues.10 In contrast, the apoB4372–4392 peptide reported in this study is far more effective than lysine analogues at inhibiting Lp(a) formation. In conclusion, this study has defined an apoB sequence (apoB4372–4392) that is important for the noncovalent binding to apo(a) in the first step of Lp(a) assembly. Furthermore, we have shown that a synthetic peptide based on this sequence is a novel inhibitor of Lp(a) formation that acts by noncovalently binding apo(a) and inhibiting the first step of Lp(a) assembly. Complexing of the peptide to DMPC vesicles enhances the inhibitory activity of the peptide. The apoB4372–4392:DMPC complex may have potential therapeutic use as an inhibitor of Lp(a) assembly that could be used to lower plasma Lp(a) levels.

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