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
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 (KYYELEEKIVSLIKNLKLKNLVLK) and a control peptide, apoB4372–4392(NM)(VKEKYSK-LILKNLLEVAYELL), 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, Tex) 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 Otago and maintained on a regular chow diet. Wild-type mouse plasma was obtained from nontransgenic littersates. 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) at 30°C in the presence of increasing amounts (1 to 400 g/mL) of the MAb a-6 antibody 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 H 2 SO 4 , 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 and apoB4372–4392(NM) peptides to inhibit apo(a) and apoB and prevent disulphide bond formation was assessed using an in vitro Lp(a) formation assay. Plasma (1 µL) from an apoB 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 apoB4372–4392(NM) control peptide or the lysine analogue e-aminono-caproic acid (e-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 e-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 NaHCO 3 , 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 quadruplicate.
Results

An alignment of the human and mouse apoB carboxyl-terminal protein sequences between amino acids 4324 and 4403 is given in Figure 1. The LALIGN program predicts an overall sequence identity of 64% between human and mouse apoB in this region. Within this are stretches of up to 10 amino acids that are perfectly conserved between mouse and human. There are 5 lysine residues in the human sequence, and 4 of these are clustered in a 21-amino acid sequence between 4372 and 4392 (highlighted in gray). Two of the four lysines (Lys4372 and Lys4385) are conserved in mouse, whereas the other two show substitutions to an asparagine at position 4372 and 4392 respectively.

The Cys4326 residue that forms the covalent linkage with apo(a) is shown along with the terminal positions of apoB95 (4330) and apoB97 (4397). Identical amino acids are indicated by capital letters. The sequence of the human apoB4372–4392 peptide is highlighted in gray.

The Cys4326 residue that forms the covalent linkage with apo(a) is shown along with the terminal positions of apoB95 (4330) and apoB97 (4397). Identical amino acids are indicated by capital letters. The sequence of the human apoB4372–4392 peptide is highlighted in gray.

Population for 1 hour at 28°C, and the plates were washed 3 times with wash buffer (0.05% Tween 20 in PBS, pH 7.4). Plates were incubated with 100 μL of hr-labeled 1D antibody diluted 1:1000 in dilution buffer for 90 minutes at 28°C. Plates were washed with wash buffer and developed with 100 μL of substrate solution (0-phenylenediamine dihydrochloride and hydrogen peroxide). Reactions were stopped by adding 100 μL of 2 mol/L H2SO4, and the absorbance of each well was read at 490 nm on a BIO-TEK EL340 plate-reader. The background absorbance was determined from incubations containing human apoB only, and this was subtracted from all data points. The percent Lp(a) formed in each incubation was calculated relative to the incubation containing no inhibitor.

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 peptide showed 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.
disruption to Lp(a) assembly (Figure 4). Complete inhibition of Lp(a) assembly was seen at 100 μmol/L peptide. Complexing of the apoB4372–4392 peptide to DMPC enhanced its inhibitory capacity, with complete inhibition of Lp(a) assembly being achieved at 40 μmol/L peptide (Figure III, available online at http://atvb.ahajournals.org). Incubations containing similar concentrations of the apoB4372–4392scram control peptide show no effect on Lp(a) formation (Figure III, available online at http://atvb.ahajournals.org).

To investigate the inhibitory capacity of the apoB4372–4392 peptide further, an Lp(a) ELISA assay was used to determine an IC50 value for the peptide both in the absence and presence of DMPC (Figure 5). For comparison, an IC50 value was also obtained for the lysine analogue ε-ACA. The apoB4372–4392 peptide proved to be an effective inhibitor of Lp(a) formation with an IC50 of 40 μmol/L. The DMPC-complexed peptide was an even better inhibitor, with an IC50 value of 4 μmol/L. The DMPC-complexed apoB4372–4392 peptide was 2500-fold more effective at inhibiting Lp(a) formation than ε-ACA, which showed an IC50 of 10 mmol/L. The IC50 value we obtained for ε-ACA was comparable with values previously reported for lysine analogues using a similar Lp(a) formation assay to the one used here.30

Discussion

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

Figure 4. Inhibition of Lp(a) formation by the apoB4372–4392 peptide. Increasing amounts of the apoB4372–4392 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.

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).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.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.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).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 apoB4372–4392 peptide bound to apo(a) with high affinity (Figure 3). Addition of the apoB4372–4392 peptide to Lp(a) formation assays showed the peptide to be an effective inhibitor of Lp(a) assembly with an estimated IC50 of 40 μmol/L (Figure 5). Complexing of the peptide to DMPC enhanced its inhibitory capacity even further, yielding an IC50 of 4 μmol/L. Interestingly, the apoB4372–4392 peptide complexed to DMPC was 2500-fold more effective at inhibiting Lp(a) formation than the lysine analogue ε-ACA, a well-known inhibitor of Lp(a) assembly.10 We propose that the apoB4372–4392 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.
amphipathic helix containing lysine residues, showed no inhibitory activity. Inhibiting Lp(a) assembly than lysine analogues, it is obvious that one or more of the apo(a) kringle IV lysine-binding pockets, facilitating the noncovalent interaction with apo(a). The Lys4372 residue is the most likely candidate, because it is the most highly conserved among species of apoB that bind apo(a) (C. Liu and S. McCormick, unpublished observations, 2002). Because the apoB4372–4392 peptide is far more effective at inhibiting Lp(a) assembly than lysine analogues, it is obvious that there are other structural features, besides the presence of lysine residues, that facilitate its interaction with apo(a). The α-helical structure of the peptide seems to be important, because stabilization of the α-helix by complexing with DMPC clearly enhanced the inhibitory capacity of the peptide. The interaction of the apoB4372–4392 peptide with apo(a) is clearly specific to this sequence, however, because recent testing of another apoB peptide spanning apoB residues 4319–4331,14 which is also predicted to form a class A amphipathic helix containing lysine residues,14 showed no inhibitory activity.

The apo(a) protein has multiple copies of the plasminogen kringle 4–like domain, of which there are 10 distinct subtypes designated KIV1 through KIV10.8 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) kringles 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, apoB3732–376539 and apoB3304–3317,40 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 IC50 to be ≈1 mmol/L, which is similar to the IC50 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.

Acknowledgments

This work was supported by grants to Dr McCormick from the Marsden Fund (contract No. UOO804) and the National Heart Foundation (grant No. 852). We thank Dr Stephen Young for the human apoB mice and for providing us with the mouse apoB partial
sequence, Dr Robert Hammer for the human apo(a) transgenic mice, and Dr Ross Milne for the 1D1 antibody.

References


A Synthetic Peptide That Inhibits Lipoprotein(a) Assembly
Rebecca J. Sharp, Matthew A. Perugini, Santica M. Marcovina and Sally P.A. McCormick

Arterioscler Thromb Vasc Biol. 2003;23:502-507; originally published online January 9, 2003;
doi: 10.1161/01.ATV.0000055741.13940.15
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://atvb.ahajournals.org/content/23/3/502

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/