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A Novel ApoC-II Mimetic Peptide that Activates LPL and Decreases Serum Triglycerides in ApoE-KO Mice

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Running title: ApoC-II mimetic peptide stimulates lipolysis

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Abbreviations: LPL, Lipoprotein Lipase; ApoC-II, apolipoprotein C-II; ApoC-III, apolipoprotein C-III; ApoA-I, apolipoprotein A-I; VLDL, Very low density lipoprotein; LDL, Low density lipoprotein; HDL, High density lipoprotein; ABCA-1, ATP-binding cassette transporter; FFA, Free fatty-acids; ACS, Acute coronary syndrome; TG, Triglycerides.

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ABSTRACT

ApoA-I mimetic peptides are currently being developed as possible new agents for the treatment of cardiovascular disease based on their ability to promote cholesterol efflux and their other beneficial anti-atherogenic properties. Many of these peptides, however, have been reported to cause transient hypertriglyceridemia due to inhibition of lipolysis by Lipoprotein Lipase (LPL). We describe a novel bi-helical amphipathic peptide (C-II-a) that contains an amphipathic helix (18A) for binding to lipoproteins and stimulating cholesterol efflux, as well as a motif based on the last helix of apoC-II that activates lipolysis by LPL. The C-II-a peptide promoted cholesterol efflux from ABCA1-transfected BHK cells similar to apoA-I mimetic peptides. Furthermore, it was shown *in vitro* to be comparable to the full-length apoC-II protein in activating lipolysis by LPL. When added to serum from a patient with apoC-II deficiency, it restored normal levels of LPL-induced lipolysis and also enhanced lipolysis in serum from patients with Type IV and Type V hypertriglyceridemia. Intravenous injection of C-II-a (30mg/kg) in ApoE-KO mice resulted at 4h in a significant reduction of plasma cholesterol and triglycerides of 38±6% and 85±7%, respectively. When co-injected with the 5A peptide (60mg/kg), the C-II-a (30mg/kg) peptide was found to completely block in C57Bl/6 mice the hypertriglyceridemic effect of the 5A peptide. In summary, C-II-a is a novel peptide based on apoC-II, which promotes cholesterol efflux and lipolysis and may, therefore, be useful for the treatment of apoC-II deficiency and other forms of hypertriglyceridemia.

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INTRODUCTION

ApoA-I mimetic peptides are short amphipathic helical peptides that are designed to mimic the biological properties of ApoA-I (Leman et al., 2014) (Gordon and Davidson, 2012) (Sethi et al., 2007) (Bielicki et al., 2010; Navab et al., 2010). In particular, these peptides have been designed to promote the cholesterol efflux from cells by the ABCA1 transporter, as well as by other mechanisms. Several of these peptides are being developed as possible therapeutic agents for the rapid stabilization of acute coronary syndrome patients based on the promising clinical trials of full-length apoA-I associated with phospholipids, which can rapidly reduce plaque size and inflammation (Krause and Remaley, 2013). ApoA-I mimetic peptides may have advantages over full-length apoA-I as a therapy based on their low cost of production, safety and route of administration (Sethi et al., 2007) (Remaley et al., 2008).

One possible limitation of apoA-I mimetic peptides, as well as full-length apoA-I, is that they have been reported to transiently cause hypertriglyceridemia (Nanjee et al., 1996; Carballo-Jane et al., 2010) (Nanjee et al., 1999) (Shaw et al., 2008). One proposed explanation for this phenomena is their ability to displace apoC-II, a known activator of Lipoprotein Lipase (LPL) (Kei et al., 2012) or they may, like apoC-III, cause direct inhibition of LPL (Shachter et al., 1994) (Jong et al., 1999) (Wang et al., 1985)) (Ooi et al., 2008). Besides apoA-I, overexpression of several different apolipoproteins have been shown to cause

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hypertriglyceridemia (Nanjee et al., 1996) (Melegh et al., 2012; Kersten, 2014). In contrast, ApoC-II is known to be one of main physiologic activators of LPL through the ability of its C-terminal helix to guide lipoproteins to the active site of LPL (Musliner et al., 1979) (Zdunek et al., 2003). In particular, amino-acid residues 63, 66, 69 and 70, in the last helix of apoC-II, have been identified as critical for LPL activation (Shen et al., 2002). Although peptides based on just the last helix of apoC-II can activate LPL when artificial lipid emulsion substrates are used, they are relatively inactive when VLDL or chylomicrons are used as a substrate, because of their poor lipoprotein binding affinity (Olivecrona and Beisiegel, 1997). Besides the last helix, ApoC-II contains two other amphipathic helices, which facilitate its binding to lipoproteins (Zdunek et al., 2003).

Hypertriglyceridemia is typically defined as triglycerides over 200 mg/dL (Berglund et al., 2012) and can be the result of several rare primary genetic defects but is more commonly due to secondary hypertriglyceridemia caused by obesity, diabetes mellitus, pregnancy, alcohol and a wide variety of drugs (Anderson et al., 2009) (Dominguez-Munoz et al., 1991). Triglycerides are enriched in chylomicrons and VLDL, and lipolysis by LPL is a critical step in the catabolism of these triglyceride-rich lipoprotein particles. Rarely hypertriglyceridemia can be due to genetic defects in LPL or apoC-II, and these patients are at risk for acute pancreatitis from their high triglycerides (Viljoen and Wierzbicki, 2012). Fibrates and fish oils are the main treatment for hypertriglyceridemia (Berglund et al., 2012), but treatment options for rapidly lowering triglycerides in patients with acute pancreatitis are limited, but include

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plasmapheresis, and intravenous (IV) infusion of heparin and/or insulin, which induces gene expression of LPL (Tsuang et al., 2009).

In the present study, we describe a novel bi-helical mimetic peptide named C-II-a, which is based on the apoC-II protein. The first two helices of apoC-II are replaced with a synthetic amphipathic peptide called 18A (Anantharamaiah et al., 1985), which has high affinity for binding to lipoproteins (Kei et al., 2012). The second helix is linked to the 18A helix by proline and contains 21 residues from the last helix of apoC-II, the LPL activating domain of apoC-II (Olivecrona and Beisiegel, 1997). We show that the CII-a peptide promotes lipolysis of triglycerides on native lipoproteins by LPL, as well as cholesterol efflux by the ABCA1 transporter. This apoC-II mimetic peptide can thus be possibly used as a new therapeutic agent for the treatment of cardiovascular disease.

MATERIAL AND METHODS

Peptide Synthesis: C-II-a (DWLKAFYDKVAEKLKEAF-P-AMSTYTGIFTDQVLSVLKGEE), and an inactive analogue called C-II-i containing four Alanine substitutions, (DWLKAFYDKVAEKLKEAF-P-AMST**A**TG**A**FT**AA**VLSVLKGEE), and an apoA-I mimetic peptide called 5A (DWLKAFYDKVAEKLKEAF- P - DWAKAAYDKAAEKAKEAA) (Sethi et al., 2008) were synthesized by a solid-phase procedure, using Fmoc protected amino acids on a Biosearch 9600 peptide synthesizer. They were purified to greater than 95% purity by reverse-phase HPLC on an Aquapore RP-300 column (Sethi et al.,

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2008), and delivered intravenously into the retro-orbital sinus. Peptides were dissolved in saline and pH was adjusted to 7.4.

Peptide Modeling and Circular Dichroism Spectroscopy: The secondary structure of the peptides was predicted, using PEPfold software (http://bioserv.rpbs.univ-paris-diderot.fr/services/PEP-FOLD/). The interaction of peptides with phospholipids was determined by coarse grain modeling, using Orientations of Proteins in Membranes (OPM) online database (http://opm.phar.umich.edu/). Depth/Hydrophobic thickness (Å), $\Delta G_{transfer}$ (kcal/mol), and Tilt angle (˚) were also analyzed using the OPM online software. The circular dichroism (CD) spectra of peptides were collected on a J-715 Spectropolarimeter (JASCO). The helicity of each peptide monitored at 222 nm were calculated by a previously reported formula (Lomize et al., 2006).

Mice and Diets: C57Bl/6 and ApoE-KO (Jackson Laboratory, Bar Harbor, ME) were fed a regular rodent chow diet (NIH-07 chow diet: 0.025% cholesterol, 4.5% fat; Ziegler Brothers, Inc., Gardner, PA). All animal studies were approved by the NHLBI Animal Care and Use Committee (protocols # H-0050, H-0018).

Analysis of Lipids and Lipoproteins: Lipids (total cholesterol, triglycerides and phospholipids) were measured enzymatically (Wako Chemicals USA, Inc. Richmond, VA) on a ChemWell 2910 analyzer (Awareness Technology, Inc, Palm City, FL). Lipoproteins were separated by fast-protein liquid chromatography (FPLC), using two Superose 6 HR 10/30 columns (Amar et al., 1998), followed by enzymatic colorimetric analysis of lipids, as described above.

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LPL Activity Assays: LPL activity was monitored with either Intralipid (2.5 ug TG per well) as the substrate or with serum (1:50 final dilution) in a reaction volume of 50 uL in PBS (pH 7.4), containing 0.1% (w/v) fatty acid free bovine serum albumin. The activity was monitored by the release of free fatty acids (FFA), as previously described (Carballo-Jane et al). 0.2 Units of LPL purified from bovine milk (Sigma, St. Louis, MO) was added per well. Recombinant full-length apoC-II protein was obtained from Meridian Life Science, Inc., TN. Free fatty acids generated during the reaction were quantified in the same plate, using commercially available reagents (Wako, Richmond, VA). Samples were read at A_{550} in a SpectraMax 384 Plus plate reader.

In Vitro **Cholesterol Efflux Assay:** Cholesterol efflux studies were performed as previously described (Remaley et al., 2003), with the following modifications. BHK-mock (control) and BHK stably transfected cells with human ABCA1 cDNA (Vaughan and Oram, 2003; Sankaranarayanan et al., 2009) were labeled for 18h with 1 mCi/mL of ^{3}H-cholesterol in DMEM media plus 10% FCS. Transporters were induced with 10nM of mifepristone in DMEM plus 0.1 mg/mL of fatty acid free BSA for 18h. Cholesterol flux was measured after the addition of media containing an acceptor plus 10nM mifepristone in DMEM with 0.1 mg/mL fatty acid free BSA. After 18h, media was collected and filtered (Whatman, 24 well 25 um pore size) and cells were lysed in 0.5mL of 0.1% SDS and 0.1N NaOH. Radioactive counts in media and cell fractions were measured by liquid scintillation counting and results are expressed as % of total counts effluxed.

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Statistical Analysis: Unless otherwise indicated, all data are expressed as mean plus minus 1 SD. *p*-values <0.05 were considered to be statistically significant when using a two-tailed T-test.

RESULTS

Design and Biophysical Characterization of C-II Peptides

Figure 1A is a diagram of the active C-II-a peptide and an inactive analogue called C-II-i. Both peptides contain two amphipathic helices linked by proline. The first or N-terminal helix, which is shared by both of these peptides, is a type A amphipathic helix based on the 18A peptide (Figure 1B) (Anantharamaiah et al., 1985). It contains a relatively high hydrophobic moment of 9.68 μH/n and is known to have high affinity for lipids and lipoproteins, and thus was used to anchor the peptides to the lipoproteins. The second helix for both peptides is based on the third and last helix of apoC-II, which is known to activate LPL (Musliner et al., 1979) (Connelly et al., 1987). This helix has a relatively low hydrophobic moment of 4.68 μH/n (Fig. 1A) and does not readily bind to lipoproteins. The inactive C-II-i peptide differs from the C-II-a active peptide in that 4 amino acid residues in the second helix, which are known to be critical in LPL activation (Shen et al., 2002) were substituted for Ala (Figure 1C).

The interactions of these peptides with phospholipids were modeled in Fig. 2. As expected, based on its relatively large hydrophobic moment, the 18A

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helix was predicted to be buried relatively deeply in the acyl chains of phospholipids in a parallel orientation to the surface (Fig. 2A). In contrast, the second helix of C-II-a, which does not form a good amphipathic helix (Fig.1A) was predicted to be only loosely associated with the outer phospholipid surface (Fig. 2B). Substitution of the 4 natural residues critical for LPL activation for Ala (Y23A, I26A, D29A and Q30A) did not substantially alter its hydrophobic moment (4.50μH/n) but increased its total hydrophobicity from 4.2 to 6.8 (Wimley–White scale) and it had a much greater predicted ΔG for lipid binding. Based on its increased ΔG and its now more circumferential arrangement of hydrophobic amino acids (Thevenet et al., 2012), it was predicted to deeply insert in phospholipid membranes in a parallel orientation to the acyl chains (Fig. 2C). These results are consistent with the analysis of the peptides by CD spectroscopy (Fig. 2D). The C-II-a and C-II-i peptides showed similar helical content when dissolved in water (data not shown), but the C-II-i peptide was much more helical (C-II-i = 100.0%; CII-a = 59.3%) in the presence of 10% TFE (Fig. 2), which simulates a lipid environment.

Stimulation of *In Vitro* Lipolysis by C-II Peptides

The ability of the C-II peptides to promote *in vitro* the lipolysis of Intralipid, a phospholipid emulsion containing triglycerides, was tested and compared to full length apoC-II and 5A, an apoA-I mimetic peptide that reduces atherosclerosis and inflammation in animal models (Dai et al., 2012) (Tabet et al., 2010) (Amar et al., 2010) but causes hypertriglyceridemia (Carballo-Jane et al., 2010).

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Increasing the concentration of C-II-a increased the generation of FFA in the presence of LPL, with the maximum effect occurring at about 0.22 umol/L, thus indicating that the peptide is a potent activator of LPL (Fig. 3). In fact, it had a very similar dose response relationship as the full-length apoC-II protein in LPL activation with an apparent Km of 7.6 nmol/L and a Vmax of 8.51 nmol/h. In contrast, both the C-II-i and the 5A peptide in the absence of any active apoC-II protein or peptide appeared to readily inhibit the production of FFA by LPL (Figure 3A) with an IC_{50} of less than 1 umol/L. In Fig. 3B, the ability of 5A to inhibit LPL was tested in the presence of maximum activating levels of C-II-a. Inhibition of LPL by 5A in the presence of C-II-a was observed at doses of 5A greater than 2.5 umol/L, and its IC_{50} for inhibiting LPL was approximately 100 umol/L.

Next, we compared the ability of the two C-II peptides to full-length apoC-II in promoting lipolysis in serum from a patient with Type IV hypertriglyceridemia and a patient with Type I hypertriglyceridemia from apoC-II deficiency (Fig. 4) (Fojo and Brewer, 1992). The addition of just LPL to serum from a patient with Type IV hypertriglyceridemia significantly increased the level of FFA and a small but statistically significant further increase in FFA generation was observed after also adding C-II-a. The addition of C-II-i along with LPL appeared to inhibit lipolysis, as there was a reduction in FFA below what was observed after just LPL addition. The addition of C-II-a or C-II-i to serum or intralipid, without first LPL, did not generate any additional production of FFA when compared to untreated samples (data not shown). Full-length apoC-II was almost as effective as the C-II-a peptide in stimulating lipolysis of LPL.

When serum from an apoC-II deficient patient was used as the substrate, no additional FFA generation was observed after the addition of LPL, confirming the necessity for LPL activation by apoC-II. But when C-II-a was added along with LPL, it stimulated the production of FFA to a similar level seen in the patient with Type IV hypertriglyceridemia. In contrast, as before, the C-II-i peptide was inactive and did not result in any further production of FFA in the presence of LPL. Full-length apoC-II behaved similar to the C-II-a peptide in restoring lipolysis to apoC-II deficient serum after LPL addition.

The effect of the C-II-a peptide was then tested in serum from patients with a wide range of hypertriglyceridemia due to Type IV and Type V hyperlipidemia (Fig. 5A). The addition of LPL to serum caused a 2-4 fold increase in the production of FFA over baseline, but the response was highly variable. Interestingly, several patients, particularly those with only a modest increase in triglycerides showed very little or no increase (see sample with TG=207 mg/dL in Fig. 5A) in FFA after the addition of LPL. The addition of C-II-a along with LPL, in all cases, however, showed additional generation of FFA above which was observed with just the LPL addition. As can be seen in Fig. 5B, the greatest percent increase in lipolysis from the addition of C-II-a was from patients with only a moderate increase in triglycerides, less than approximately 500 mg/dL. Next, the ability of C-II-a to stimulate lipolysis in hypertriglyceridemic serum (TG<500 mg/dL) above what was observed with just LPL addition was compared to full- length apoC-II protein in Fig. 5C. Both increased lipolysis after LPL

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addition, but C-II-a treatment showed for all samples a higher rate of lipolysis than full-length apoC-II treatment and on average resulted in a more than 2-fold increase in FFA levels above baseline compared to the full-length apoC-II protein.

Stimulation of *In Vitro* Cholesterol Efflux by C-II Peptides

The C-II peptides and the 5A peptide, which has been shown to mediate cholesterol efflux by the ABCA1 transporter (Sethi et al., 2007), both share the first 18A helix. Like the C-II-a peptide, the second helix of the 5A peptide has a relatively low lipid binding affinity. Its second helix is a modified 18A helix containing 5 Ala substitutions for hydrophobic residues, which reduces its lipid affinity but has been shown to improve its specificity for cholesterol efflux by the ABCA1 transporter and to reduce cytotoxicity (Sethi et al., 2007). We, therefore, compared the ability of the C-II peptides and the 5A peptide in promoting cholesterol efflux from ABCA1 transfected cells (Fig. 6A). The 5A peptide showed the most cholesterol efflux from ABCA1 transfected cells, but both the C-II-a and the C-II-i peptides also showed considerable specific efflux from ABCA1 transfected cells above that observed with the mock transfected cells (Fig. 6B).

Stimulation of *In Vivo* Lipolysis C-II Peptides

Next, we examined in Fig. 7 the ability of the C-II-a peptide to promote lipolysis in apoE-KO mice. Intravenous injection of C-II-a caused a rapid reduction in serum triglycerides. By 4h, C-II-a reduced serum triglycerides by

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approximately 85% compared to baseline (Fig. 7A). Total cholesterol also showed a similar trend as triglycerides but showed less of a change with a maximum decrease of 38% at 4h (Fig. 7B). 4h after injection of the peptide, plasma lipids were also analyzed by FPLC analysis (Fig. 7C, 7D). Based on this analysis, the majority of the decrease in triglycerides and cholesterol occurred on VLDL and to a lesser degree on LDL. In contrast, the C-II-i peptide caused hypertriglyceridemia, with most of the changes due to increase triglycerides on VLDL (Fig. 7E). Total cholesterol did not show a major change following C-II-i treatment (Fig. 7F).

In Fig. 8, we examined the effect of intravenously injecting C57Bl/6 mice with 5A with and without the C-II-a peptide on serum triglycerides. The 5A peptide caused a marked but transient increase in triglycerides, whereas the C-II-a peptide by itself had no significant effect on triglycerides compared to the baseline or saline control. When both the 5A and C-II-a peptides were coinjected into mice, the C-II-a peptide blocked the increase in triglycerides observed when the 5A peptide was injected alone. This suggests that the C-II-a peptide by promoting lipolysis can prevent hypertriglyceridemia from the 5A apoA-I mimetic peptide treatment.

DISCUSSION

Recently there has been great interest in the development of apolipoprotein mimetic peptides for cardiovascular disease and also a wide variety of other disorders related to inflammation and Alzhemier's disease (Sethi

et al., 2007) (Navab et al., 2010) (Bielicki et al., 2010), but most of these peptides are based on sequences derived from apoA-I and apoE. In this report, we describe a novel apolipoprotein mimetic peptide based on apoC-II, which is an important physiologic activator of lipolysis by LPL. It was already known that the last helix of apoC-II was critical in the activation of LPL and 4 amino acid residues critical in this process have been mapped to the last helix of apoC-II (Shen et al., 2002). Most of these amino acid residues are near the polar face of the helix and are thought to possibly interact with LPL and tether it to the surface of lipoproteins where it can interact with its triglyceride substrate. Although the last helix of apoC-II by itself can activate LPL when using artificial lipid emulsions as a substrate, it is inactive with natural lipoprotein substrates because of its poor affinity for lipoproteins (Olivecrona and Beisiegel, 1997). To address this issue, we attached the last helix of apoC-II to the 18A helix, which has been previously used in the design of other apolipoprotein mimetic peptides, because of its high lipid binding affinity (Sethi et al., 2008). The resulting bi-helical peptide called C-II-a was considerably shorter than full-length apoC-II protein but similar in its ability to activate LPL with both intralipid emulsions (Fig. 3) and with natural lipoprotein substrates (Fig. 5). C-II-i, the inactive version of the peptide, was not only ineffective in activating LPL, but, in fact, inhibited LPL activity (Fig. 3A). It did so even in the absence of any active C-II-a peptide or full-length apoC-II protein (Fig. 3), so it may perhaps directly inhibit LPL by changing the lipid interface and or possibly by competing with LPL for lipid binding. The predicted orientation of C-II-i in lipid surface was much different than C-II-a (Fig. 2), which could possibly account for its ability to act as a LPL inhibitor.

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It is also possible that other mechanisms independent of LPL lipolysis may also play a role, at least partially, on the observed *in vivo* reduction of triglycerides in mice by the C-II-a peptide. It was recently reported that apoC-III inhibition by ASO in Type I patients with defective LPL unexpectedly lowered serum triglycerides (Graham et al., 2013). This may be possibly related to the role of apoC-III in inhibiting other lipases, such as hepatic lipase or endothelial lipase, whereas apoC-II and possibly the C-II-a peptide could, in contrast, stimulate lipolysis by activating other lipases. Alternatively, apoC-III may possibly be interfering with hepatic uptake of remnant lipoproteins (Graham et al., 2013). It has been shown that catalytically inactive LPL can, in fact, promote the hepatic uptake of remnant lipoproteins by acting as an alternative ligand for binding to hepatocytes (Gonzalez-Navarro et al., 2004). ApoC-III could possibly interfere with this process, whereas apoC-II and the C-II-a peptides by promoting the interaction of LPL with remnant lipoproteins could facilitate this process and hence accelerate hepatic clearance of remnant lipoproteins independent of lipolysis. These other possible mechanisms of action of the C-II-a peptide on triglyceride metabolism will be investigated in future studies.

Our initial objective for developing apoC-II mimetic peptides was to investigate and possibly address the hypertriglyceridemia caused by apoA-I mimetic peptides (Carballo-Jane et al., 2010). ApoA-I mimetic peptides were first developed as structural probes of apolipoproteins (Anantharamaiah et al., 1985) but were then discovered to share many of the beneficial anti-atherogenic properties of apoA-I. Recently, some of these peptides have been tested in early stage clinical trials as alternatives to full-length apoA-I for the rapid stabilization

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of patients with acute coronary syndrome (ACS) (Krause and Remaley, 2013). The 5A peptide like other apoA-I mimetic peptides can acutely raise triglycerides quite significantly (Fig. 8), which appears to be due to inhibition of LPL (Fig. 3). In contrast to 5A, we observed that the C-II-a peptide activated LPL with similar efficiency as the full-length apoC-II protein, using both artificial lipid emulsion substrates (Fig. 3), as well as with lipoprotein substrates (Fig. 4, 5). The C-II-a peptide also was quite effective in promoting cholesterol efflux by ABCA1 (Fig. 6), and thus possibly could be used like 5A and other apoA-I mimetic peptides as a treatment for ACS without the perhaps the dose-limiting effects from hypertriglyceridemia. Alternatively, as shown in Fig. 8, C-II-a could possibly be used in conjunction with 5A and other apoA-I mimetic peptides for blunting their hypertriglyceridemic effect.

The newly described C-II-a peptide has some other potential therapeutic indications. Genetic deficiency of apoC-II is relatively rare cause of Type I hyperlipidemia but like genetic defects due to mutations in LPL, these patients can develop remarkably elevated levels of plasma triglycerides, which can lead to acute pancreatitis (Viljoen and Wierzbicki, 2012) (Fojo and Brewer, 1992). Currently, there is no specific therapy for such patients but given the normaliztion of lipolysis that occured after adding the peptide to plasma from a patient with apoC-II deficiency, the C-II-a peptide may be useful as a therapy for this disorder. Based on the results shown, it would be predicted to rapidly lower plasma triglycerides in apoC-II deficient patients, if given during acute pancareatitis, which would be expected to help in its resolution (Viljoen and Wierzbicki, 2012) (Tsuang et al., 2009). If a convenient formulation could be developed so that it could be

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deliverd subcutaneously or orally, it could perhaps also be used prophylacticaly in these patients for preventing the initial development of hypertriglyceridemia and the triggering of pancreatitis.

Interestingly, we observed that the addition of the C-II-a peptide to patients with more common forms of hypertriglyceridemia also showed increased lipolysis after the addition of LPL (Fig. 5). Besides apoC-II and apoA-V (Fruchart-Najib et al., 2004), the other known protein activator of LPL, many other apolipoproteins are known to inhibit LPL (Kersten, 2014), particularly apoC-III. It may be that the ratio of activator to inhibitor proteins on lipoproteins determines their overall rate of lipolysis by LPL and the presence of additional activator proteins in the form of the C-II-a peptide may help promote lipolysis. Interestingly, we observed that many patients with only modest inceases in triglycerides appeared to be relatively resistant to lipolysis after adding LPL but this was relieved after adding the C-II-a peptide (Fig. 5). This possibly suggests that intermediate or small remnant-like particles may be relatively depleted in apoC-II or perhaps enriched in apoC-III and that the C-II-a peptide could also be potentially useful for more common forms of hypertriglycerimia. It is a rare complication, but some patients with Type IV and V hypertriglyceridemia can also develop pancreatitis and thus could perhaps also benefit from an apoC-II mimetic peptide therapy. Recently, hypertriglyceridemia has also been recognized as an important independent risk factor for cardiovascular disease. From GWAS studies, many of the genes related to triglyceride metabolsim have been associated with myocardial disease (Musunuru et al., 2010). For example, mutations in the apoC-III gene leading to low plasma levels of triglycerides have

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been associated with markedly lower cardiovascular disease risk (Pollin et al., 2008). Polymorphisms in apoC-III gene have also recently been found to be closely linked to cardiovascular disease (Tg et al., 2014). These findings suggest that strategies for lowering serum triglycerides perhaps by apoC-II mimetic peptides could also be a useful approach for decreasing cardiovascular disease risk in the general population with hypertriglyceridemia.

In summary, we describe a new class of apoliprotein mimetic peptides based on apoC-II. The novel apoC-II mimetic peptide called C-II-a is a potent activator of LPL and lowers serum triglycerides. Additional studies are needed, but the current results suggest that the C-II-a peptide and/or related apoC-II mimetic peptides may have several clinical indications for the treatment and prevention of cardiovasular disease.

Authorship Contribution:

Participated in research design: Amar, Remaley

Conducted experiments: Amar, Sakurai, Sviridov, Freeman, Ahsan, Sakurai-Ikuta

Performed data analysis: Amar, Sakurai, Sviridov, Freeman, Ahsan, Sakurai-Ikuta

Wrote or contributed to the writing of the manuscript: Amar, Remaley

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FIGURE LEGENDS

Figure 1. Helical wheel and net plots of ApoC-II mimetic peptides. A. Helical wheel plot of the 3rd helix of apoC-II. Arrows show site of Ala substitutions for C-II-i peptide. Size of balls and color indicate degree of charge and hydrophobicity (yellow=nonpolar, green=polar/uncharged, pink=acidic, blue=basic) B. Helical wheel plot of 18A helix. C. Helical net plot of C-II-a peptide. Central region indicates position of hydrophobic face. Arrows show sites of Ala substitutions for C-II-i peptide. First helix (1-18) is based on 18A helix. Second helix (20-40) is based on the third helix of apoC-II.

Figure 2. Structure models of ApoC-II mimetic peptides. A-C. Structural models for the binding of 18A (A), 3rd helix apoC-II (B) and Ala-substituted 3rd helix apoC-II (C) are shown. Red circles represent boundary between phospholipid headgroups (out) and acyl chains below (in). Lipid binding parameters are listed below. (**D**) CD spectrum of indicated peptides in 10% TFE.

Figure 3. Effects of peptides and ApoC-II on lipolysis of intralipid by LPL. (**A**) Intralipid, containing 2.5 ug of TG per well, were mixed with phosphate buffer saline containing 0.1% (w/v) bovine serum albumin, 0.2 Units of LPL from bovine milk and the indicated amounts of C-II-a (O), C-II-I (X), 5A (∆) or ApoCII (●) into the well of a 96-well microtiter plates. Free fatty acids were measured in the same plate using an enzymatic assay. (**B)**. The inhibition of lipolysis by LPL was

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monitored, using the above assay conditions, with 6 umol/L of C-II-a in each well and the indicated concentration of 5A. Baseline FFA values in absence of 5A peptide was 8.5 nmol. Results represent the mean of triplicates ± S.D.

Figure 4. Effect of peptides on *in vitro* lipolysis of serum. Serum from a hypertriglyceridemic patient (TG=302mg/dL) or serum from an apoC-II deficient patient (TG=638 mg/dL) were incubated, *in vitro*, with LPL alone or in conjunction with C-II-a (10 umol/L), C-II-I (10 umol/L) or full-length human apoC-II protein and the appearance of FFA was measured. Results represent the mean of triplicates ± S.D. *p<0.05 compared to serum baseline, **p<0.05 compared to serum+LPL, #p<0.05 compared to serum+LPL+C-II-a group.

Figure 5. Effect of peptides on lipolysis in serum. (A) A wide range serum samples from 20 hypertrigliceridemic patients (TG=207-716 mg/dL) were incubated, *in vitro*, with LPL alone (0.2U) or LPL + C-II-a peptide (10 umol/L) and the appearance of FFA were measured. Representative results from 6 patients are shown. (B) Mean % change in FFA after addition of LPL (●) compared to baseline (no LPL) or LPL + C-II-a peptide (▲) compared to LPL only baseline in hypertriglyceridemic serum. (C). Mean % change in FFA after addition LPL and C-II-a peptide (50 umol/L) or full-length apoC-II protein (10 umol/L) compared to LPL only baseline for hypertriglyceridemic serum samples (n=9) with a mean TG of 314 mg/dL (range 224-436 mg/dL). Results represent the mean of triplicates ± S.D., p<0.05 compared to baseline.

Figure 6. Effect of peptides on cholesterol efflux. Cholesterol efflux was measured on BHK-ABCA1 (A) and control BHK cell line (4B) after 18 h at 37 °C with the following peptides at the indicated doses: 5A (gray lines), C-II-i (dotted lines) or C-II-a peptide (black lines). Results represent the mean of triplicates ± S.D. *p<0.05 compared to baseline.

Figure 7. Effect of ApoC-II mimetic peptides on lipids in ApoE K/O mice. Apo E K/O mice (female, n= 3 per group) received an IV bolus injection of C-II-a or C-II-i peptide (30mg/kg). Retro-orbital bleedings were performed up to 4h post-injection. FPLC separation and lipid analysis were performed on pooled samples for triglycerides (Figures 7A, 7C, and 7E) and cholesterol (Figures 7B, 7D and 7F). Results represent the mean of triplicates ± S.D. *p<0.05 compared to baseline.

Figure 8. Effect of coadmininstration of C-II-a along with 5A on hypertriglyceridemia in mice. C57Bl/6 mice (female, n= 3 per group) received either a 2mg (60mg/kg) bolus of IV injection of 5A (dotted line), 1mg (30mg/kg) of C-II-a peptide co-injected with 2mg of 5A peptide (gray line), or just 1mg (30mg/kg) of the C-II-a (black line). Results from saline injected mice are shown as a black dotted line. Plasma was collected from retro-orbital sinus and

analyzed for triglycerides. Results represent the mean of triplicates ± S.D, *p<0.05 compared to saline baseline.

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Figure 1

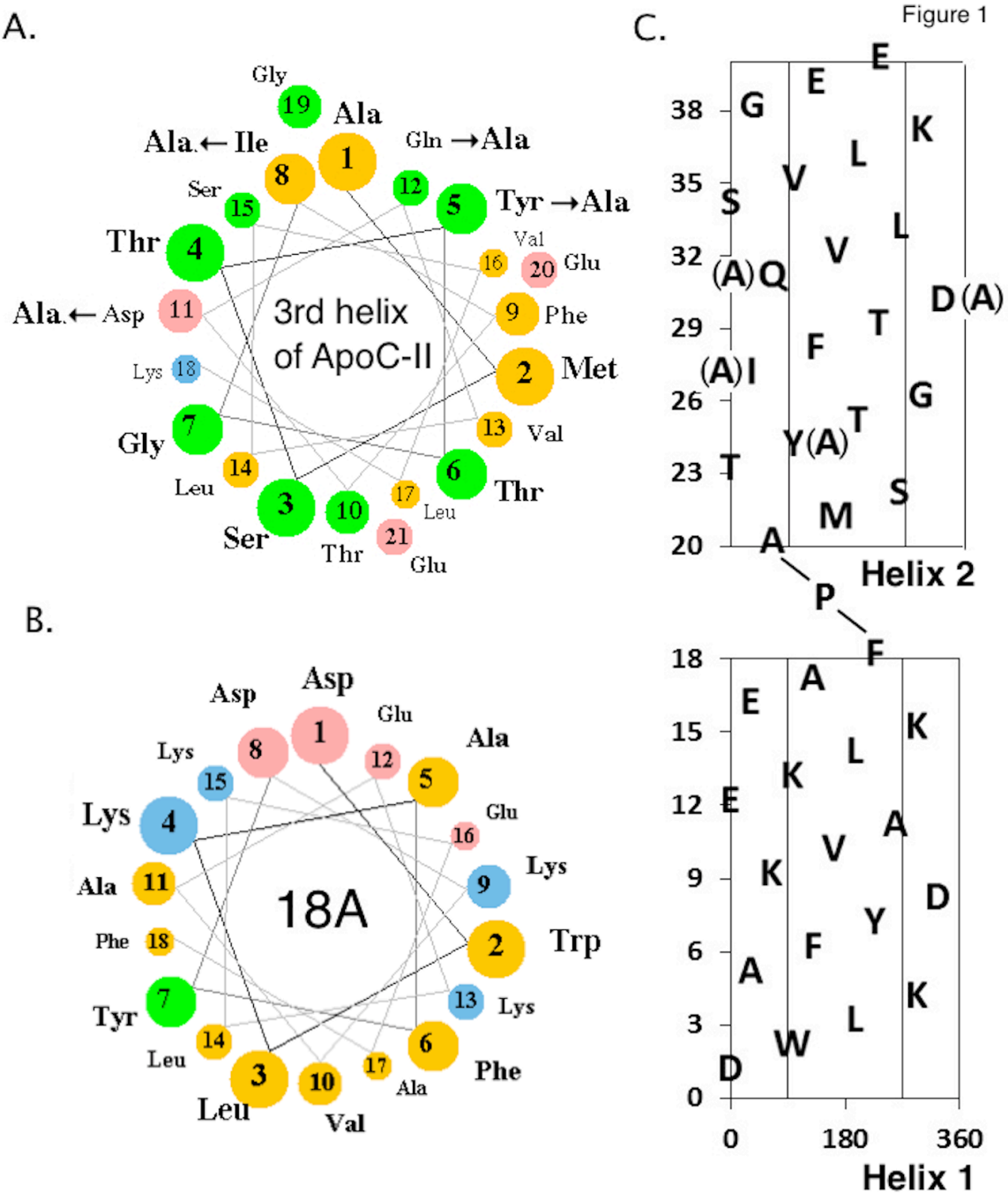

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Figure 2

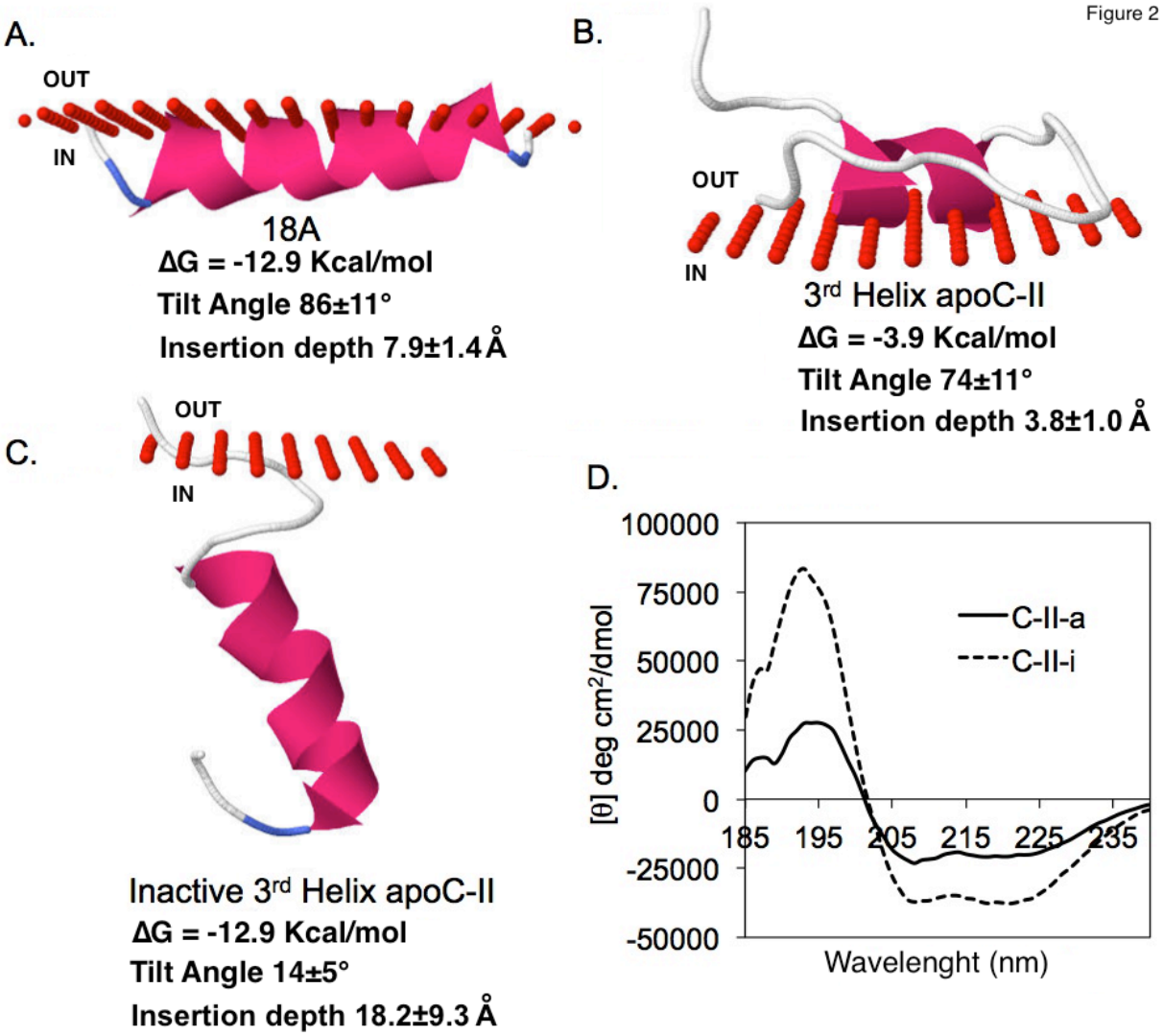

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Figure 3

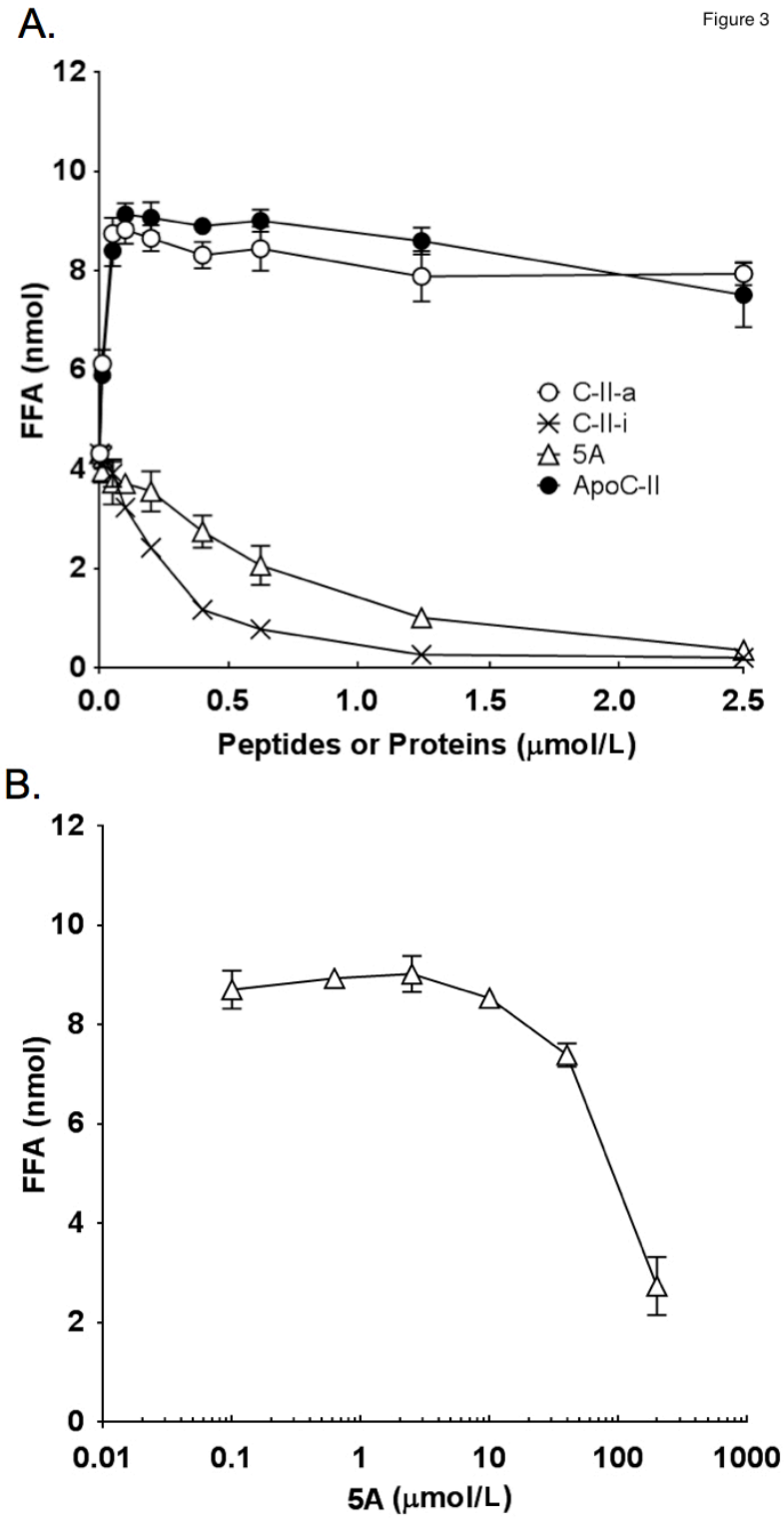

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Figure 4

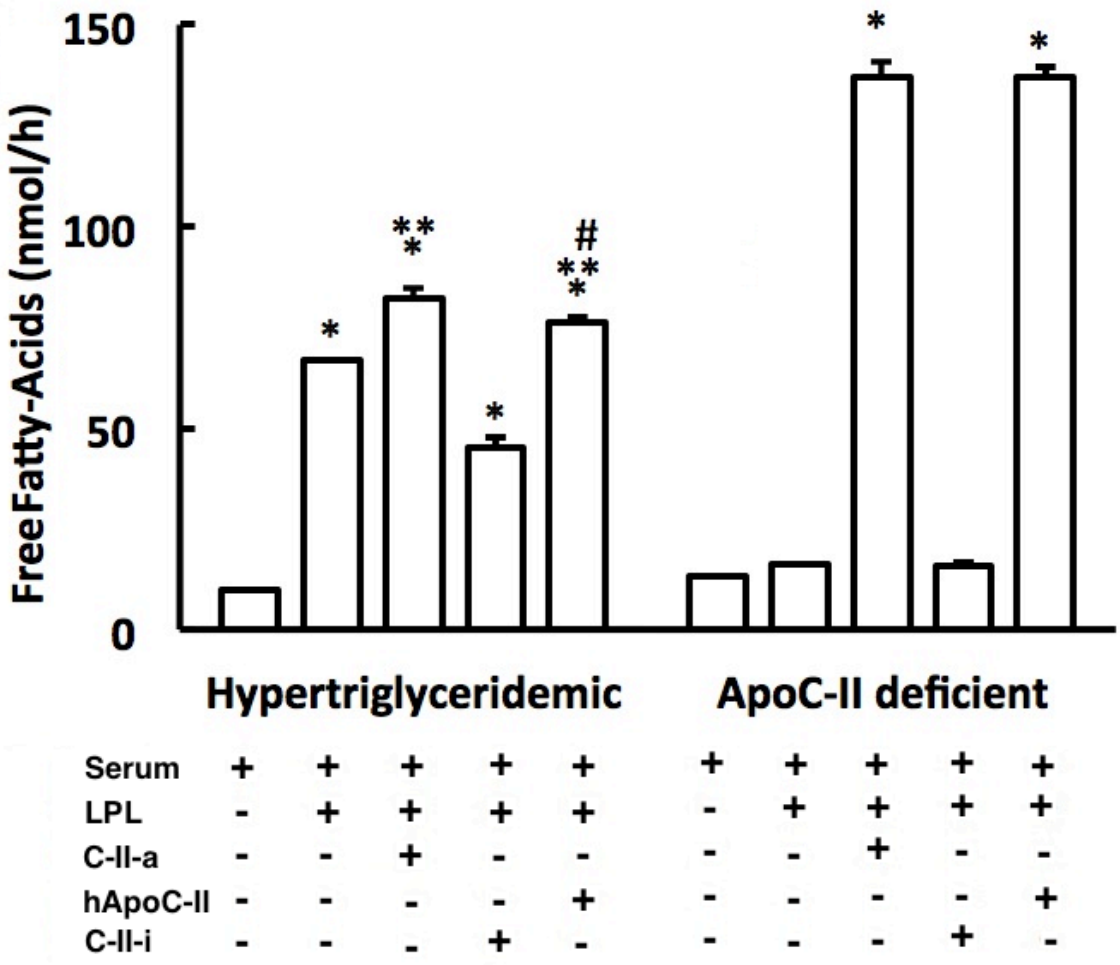

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Figure 5

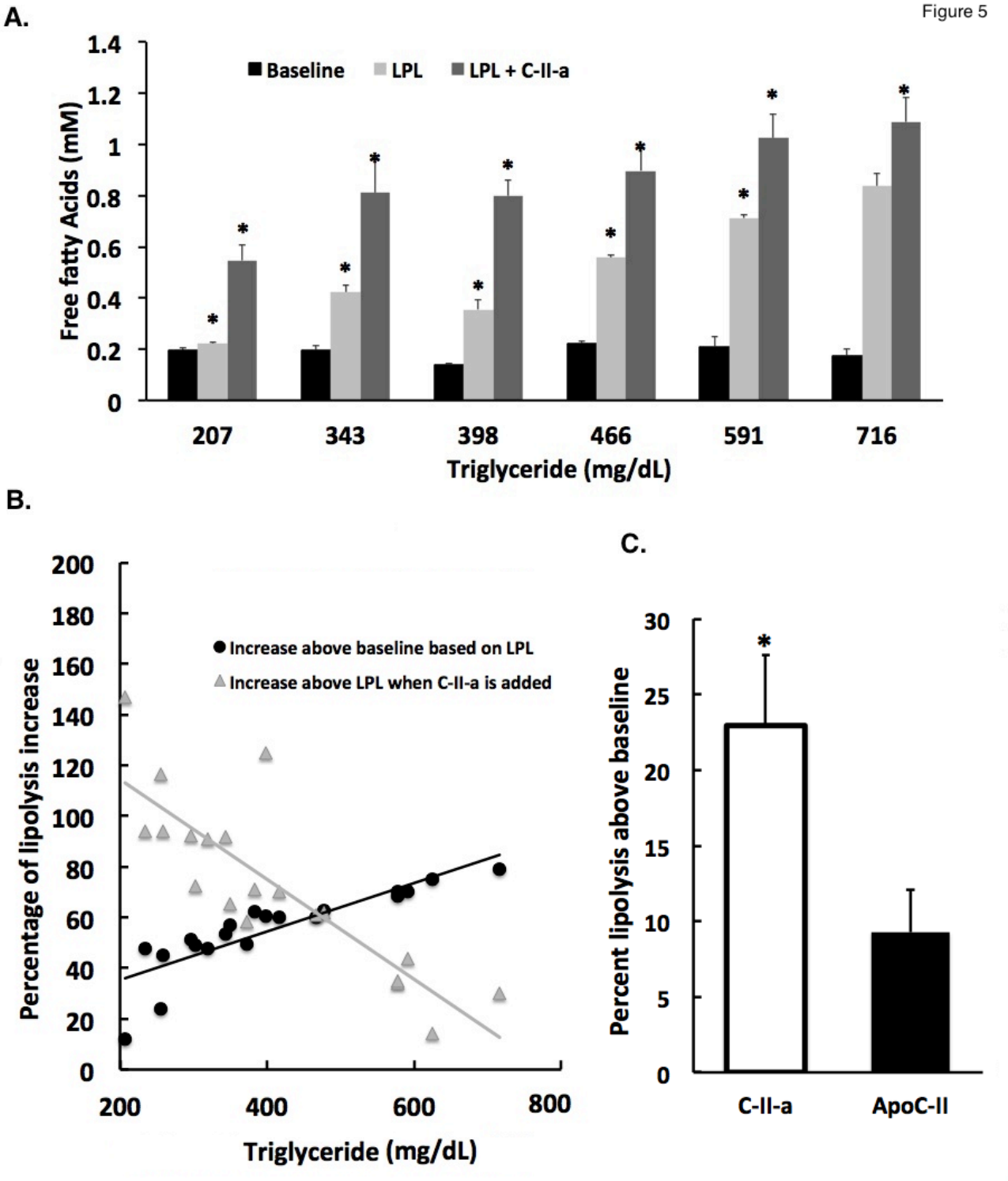

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Figure 6

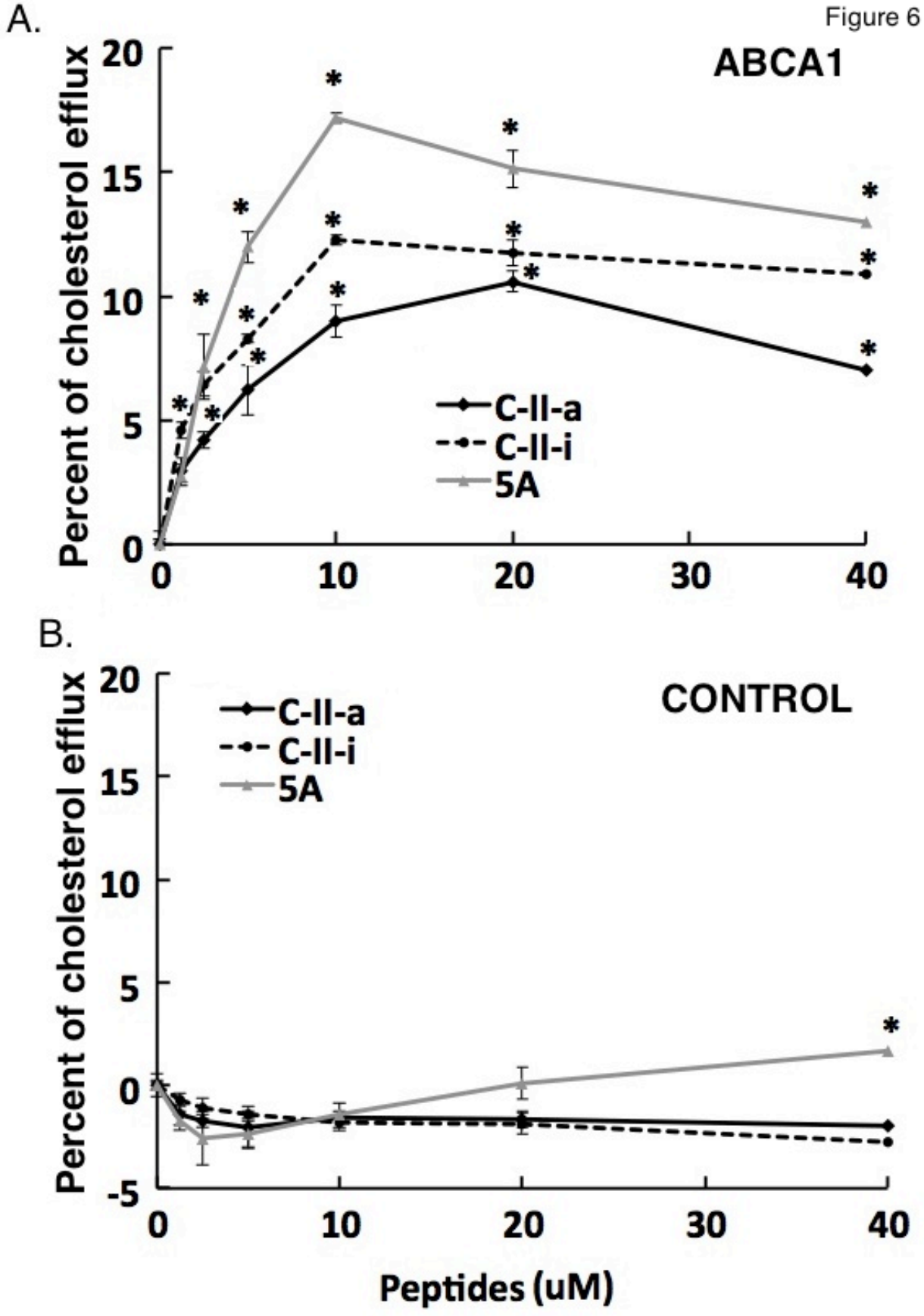

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Figure 7

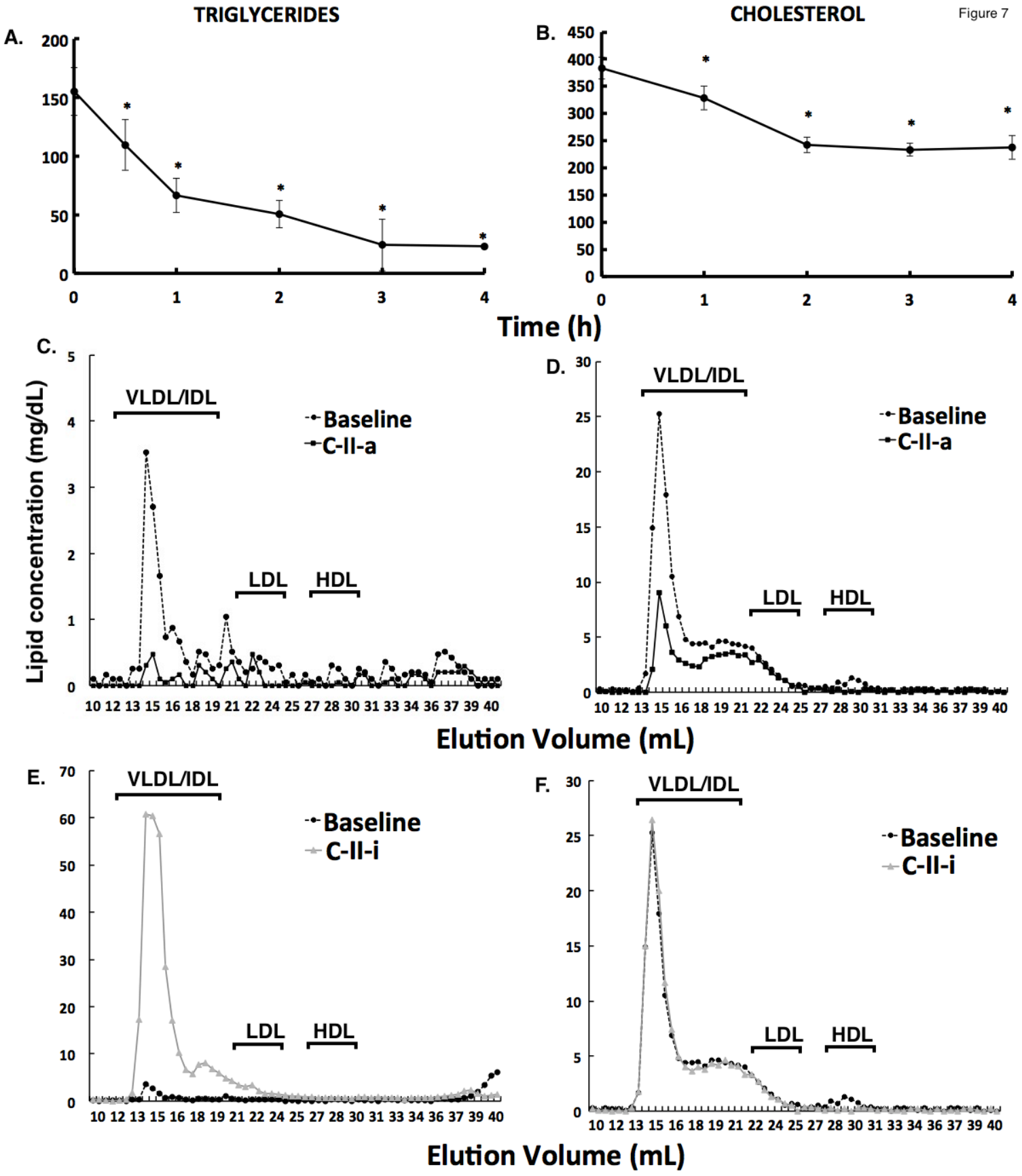

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Figure 8