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Apolipoprotein A-I attenuates LL-37-induced endothelial cell cytotoxicity

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ABSTRACT

The human cathelicidin peptide LL-37 has antimicrobial and anti-biofilm functions, but LL-37 may also damage the host by triggering inflammation and exerting a cytotoxic effect, thereby reducing host cell viability. Human plasma mitigates LL-37-induced host cell cytotoxicity but the underlying mechanisms are not completely understood. Apolipoprotein A-I (ApoA-I) is a plasma protein endowed with atheroprotective effects. Here, we investigate the interaction between ApoA-I and LL-37 by biochemical techniques, and furthermore assess if ApoA-I protects against LL-37-evoked cytotoxicity in human umbilical vein endothelial cells (HUVEC). Our results demonstrated that ApoA-I effectively binds LL-37. The binding of ApoA-I to LL-37 resulted in a structural rearrangement of the protein, but this interaction did not cause lower ApoA-I stability. Recombinant ApoA-I protected against LL-37-induced cytotoxicity in HUVEC and endogenous ApoA-I knockdown in HepG2 cells made the cells more sensitive to LL-37-evoked cytotoxicity. We conclude that ApoA-I physically interacts with LL-37 and antagonizes LL-37-induced down-regulation of endothelial cell viability suggesting that this mechanism counteracts endothelial cell dysfunction.

1. Introduction

LL-37 is synthesized as a pro-protein, named hCAP18, by blood cells such as granulocytes, lymphocytes and monocytes. hCAP18 is secreted from the cells and processed to LL-37 by proteinase 3. In its active form, LL-37 has high electrostatic and hydrophobic affinity to cell membranes. Upon binding, LL-37 may cause pore formation, regardless if the membrane belongs to an invading pathogen or the host cells themselves [1]. Indeed, LL-37 has been shown to reduce cell viability in many different human cell types [2,3].

Circulating plasma levels of hCAP18 are high in healthy individuals, 7.4 ± 0.13 μM [4]. However, expression of hCAP18 is increased in atherosclerotic lesions. Here, hCAP18 is synthesized by accumulating macrophages, neutrophils and endothelial cells, resulting in a six-fold increase of its expression compared to normal arteries [5]. Indeed, LL-37 has been implicated in the pathogenesis of atherosclerosis [6]. The vascular endothelium faces the blood and represents a critical organ for maintaining cardiovascular homeostasis. Endothelial cells regulate transport between blood and tissues and produce vasoactive factors such as NO and endothelin which control vascular tone and blood pressure. Loss of endothelial function is believed to be a key event in the development of atherosclerosis [7]. Data on deleterious effects of LL-37 on endothelial cell viability have, to the best of our knowledge, not been presented before.

Fortunately, the above mentioned harmful effects exerted by LL-37 on host cell viability are limited by endogenous proteins, such as mucin I in saliva and gC1qR/p33 in keratinocytes, which bind and inactivate the peptide [2,8,9]. Human plasma is also known to reduce LL-37's activity [3] but the scavenger involved and the underlying mechanisms are still not completely understood [10,11]. Apolipoprotein A-I (ApoA-I) is an abundant plasma protein (50 μM) and the major protein constituent of high density lipoprotein (HDL) [12,13]. ApoA-I is known to possess anti-inflammatory and antioxidant activities [14] and, more recently, it has been found to have a positive effect on glucose homeostasis [15,16]. HDL-bound ApoA-I is also the key molecule for the removal of cholesterol from peripheral tissues and its catabolism. Because of its involvement in this cellular mechanism, known as reverse cholesterol transport, ApoA-I is considered an atheroprotective molecule [17]. In addition, ApoA-I was reported to bind LL-37 and to inhibit the peptide's antibacterial effects but it has not been clarified if ApoA-I

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also antagonizes LL-37-evoked host cell cytotoxicity [10,12,13].

Here, we demonstrate that ApoA-I physically interacts with LL-37 and show for the first time that ApoA-I protects from LL-37-induced cytotoxicity in human endothelial cells.

2. Materials and methods

2.1. Production of recombinant ApoA-I

ApoA-I was produced in an *Escherichia coli* strain BL21 (DE3) pLysS (Invitrogen) and purified to homogeneity by the methods previously described in Ref. [18].

2.2. Spectroscopic analyses

ApoA-I (3 μM) was incubated in PBS for 30 min with increasing concentrations of LL-37 (Bachem), at protein to peptide molar ratios as indicated in Figs. 1 and 4 and S1. At the end of incubation, intrinsic fluorescence and Circular Dichroism (CD) spectra of ApoA-I/LL-37 mixture were recorded.

2.2.1. Intrinsic fluorescence analysis

Emission intrinsic fluorescence measurements were carried out at 25 °C in a 10-mm cell by using a Jasco J-810 spectropolarimeter equipped with a FMO-427S fluorescence module. Spectra were recorded in the range 300–450 nm, following excitation at 280 (tyrosine/tryptophan) or 295 nm (tryptophan), with a scan speed of 150 nm/min and 5 nm slit width. Maximum emission fluorescence wavelength (λ_{max}) was then plotted as a function of protein to peptide molar ratio.

2.2.2. Circular Dichroism (CD) spectroscopy and K_d determination

CD spectra were acquired on a Jasco J-810 spectropolarimeter equipped with a Jasco CDF-426S Peltier, set to 25 °C. Samples were loaded into a 1 mm quartz cuvette and CD spectra acquired at 25 °C in the far-UV range 190–260 nm, with a 1 nm wavelength increment. To estimate the specific contribution of ApoA-I to the CD signal, the spectra of the peptide alone were subtracted at the spectra of the mixture (see Figure S1). The CD signal at 222 nm for each protein to peptide molar ratio was plotted as a function of peptide concentration and the experimental data were fitted by using one-site binding equation of non-linear regression, in GraphPad Prism 7, to calculate the K_d.

2.3. Thermal denaturation analysis

ApoA-I thermal denaturation was performed, either in the absence or in the presence of LL-37 (protein to peptide ratio 1:2), by intrinsic fluorescence and CD. Intrinsic fluorescence thermal unfolding was performed as described in Ref. [19]. Briefly, samples were incubated at each temperature for 15 min (between 25 and 95 °C with 5 °C increment) before acquiring the spectra. Melting curves were obtained by reporting the λ_{max} as a function of temperature. Thermal denaturation followed by CD spectroscopy was performed by recording protein signal at 222 nm between 25 and 95 °C with 2 °C increment. In both cases, the experimental data were fitted and melting temperature (Tm) estimated by sigmoidal fitting using GraphPad Prism 7.

2.4. Cells and cell culture

Primary human umbilical vein endothelial cells (HUVEC) were purchased from Lonza and cultured in complete EGM-2 medium (Lonza) according to manufacturer's instructions. Human hepatocellular carcinoma cells (HepG2) were obtained from ATCC and cultured in DMEM/Ham's F12 medium supplemented with antibiotics and 10% FBS. The cells were kept in a water-jacketed cell incubator at 5% CO2 in air at 37 °C and trypsinized (0.25% trypsin, 0.02% EDTA) upon reaching confluence.

2.5. Downregulation of ApoA-I expression

ApoA-I expression was transiently suppressed in HepG2 hepatocytes using siRNA. Briefly, a mixture of ApoA-I siRNA (Hs_ApoA1_4 and Hs_ApoA1_7, 20 nM each, both from Qiagen) or a non-targeting control (Negative Control siRNA, 40 nM, Qiagen), were prepared in Opti-MEM medium (Thermo Fisher Scientific) together with Oligofectamine transfection reagent, according to manufacturer's instructions (Invitrogen). Cells were then seeded into the siRNA mixture at a density of 10 000 cells/cm^2 in volumes of 40 μl or 400 μl/well for 96 and 12 well plates, respectively. After 24 h of transfection, cultures were supplemented with an equal volume of DMEM containing 2% FBS (1% final concentration) and incubated for 3 days before the analyses.

2.6. Cell viability assay

Cell viability was assessed in HUVEC and HepG2 cells cultured in 96 well-plates by the MTT assay. Experiments on HUVEC were performed in EGM-2 medium without FBS, while HepG2 cells were treated in end-stage transfection medium (see paragraph 2.5). The cells were pre-treated with ApoA-I or PBS (vehicle) for 15 min before administration of LL-37 in PBS. After 3 h of treatment with peptide, cells were incubated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 0.5 mg/ml, Sigma Aldrich) for 1 h. After removal of medium the formazan, which is formed by viable cells, were dissolved in DMSO and quantified by its absorbance at 540 nm using a Multiscan GO Microplate Spectrophotometer (Thermo Scientific).

2.7. Western blotting

Relative ApoA-I protein expression was determined by western blotting as previously described [20]. Conditioned cell media were collected and cells were lysed and proteins extracted in an SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol). Total protein content was determined by the Bio-Rad DC Protein Assay. Aliquots corresponding to 30 μg of cytosolic proteins (cell lysate) or 20 μl of conditioned medium were analysed by SDS-PAGE using Criterion TGX Any kD precast gels (Bio-Rad) following reduction by 2-mercaptoethanol (10%, v/v). Standards of recombinant ApoA-I were run in parallel to samples on some of the gels. After separation, proteins were transferred to 0.2 μm nitrocellulose membranes by a *Trans*-Blot Turbo transfer system (Bio-Rad). Membranes were blocked in 1% casein (w/v) in TBS (Bio-Rad) and incubated overnight with anti-ApoA-I (Abcam, ab64308, 1:1000 dilution) and anti-GAPDH antibodies (Merck Millipore, clone 6C5, 1:5000 dilution), used as housekeeping protein. Immunoreactive bands were visualized by chemiluminescence using HRP-conjugated secondary anti-rabbit or anti-mouse antibodies, followed by incubation with SuperSignal West Femto chemiluminescence reagent (Thermo Fisher Scientific). Images were acquired using a LI-COR Odyssey Fc instrument (LI-COR Biosciences).

3. Results and discussion

3.1. ApoA-I specifically binds LL-37

Protein-protein binding and interaction is often accompanied by

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a conformational rearrangement of the species involved. To demonstrate the binding between LL-37 and ApoA-I and to study the impact of the binding on the overall protein structure, intrinsic fluorescence and CD spectra were acquired at increasing protein to peptide molar ratios.

Protein intrinsic fluorescence is due to the ability of tyrosine and tryptophan residues to emit fluorescence when excited at specific wavelengths. ApoA-I contains five tyrosine (at positions 18, 29, 100, 115, 166) and four tryptophan (at positions 8, 50, 72 and 108) residues. Changes in solvent exposure of these residues, which are reflected in changes in the maximum emission wavelength (λ_{max}), are diagnostic of protein conformational rearrangement. Fluorescence spectra were acquired when exciting only tryptophan (Fig. 1a, left panel), or both tyrosine and tryptophan residues (Fig. 1a, right panel), and λ_{max} was plotted as a function of protein to peptide molar ratio. The addition of LL-37 at increasing concentrations to ApoA-I led to a significant increase of λ_{max} that reached a plateau at a protein to peptide molar ratio of about 1:1. This observation suggests that ApoA-I specifically binds LL-37, probably at a 1:1 stoichiometry, and that this binding alters ApoA-I conformation. Since all tyrosine and tryptophan residues are located in the four-helix bundle domain of ApoA-I [21], which mostly includes the N-terminal part of the protein, it is conceivable that LL-37 binding involves a N-terminal domain structural rearrangement of ApoA-I.

LL-37 binding to ApoA-I also imposed changes in protein secondary structure, as demonstrated by changes in the CD spectra of the protein in the presence of increasing amounts of LL-37 (Fig. 1b, left panel and Fig. S1). The fitting of the CD signals at 222 nm allowed for estimation of the K_d (Fig. 1b, right panel), which was determined to 0.82 ± 0.59 μM.

These results are in good agreement with previous reports indicating that ApoA-I is able to bind LL-37 with a 1:1 stoichiometry and with a K_d in the micromolar range [12]. Interestingly, it has been suggested that ApoA-I-bound LL-37 may act as a reservoir of LL-37 to be released during infection to combat pathogens [11]. The moderate strength of interaction between the ApoA-I and LL-37 molecules is in line with this hypothesis.

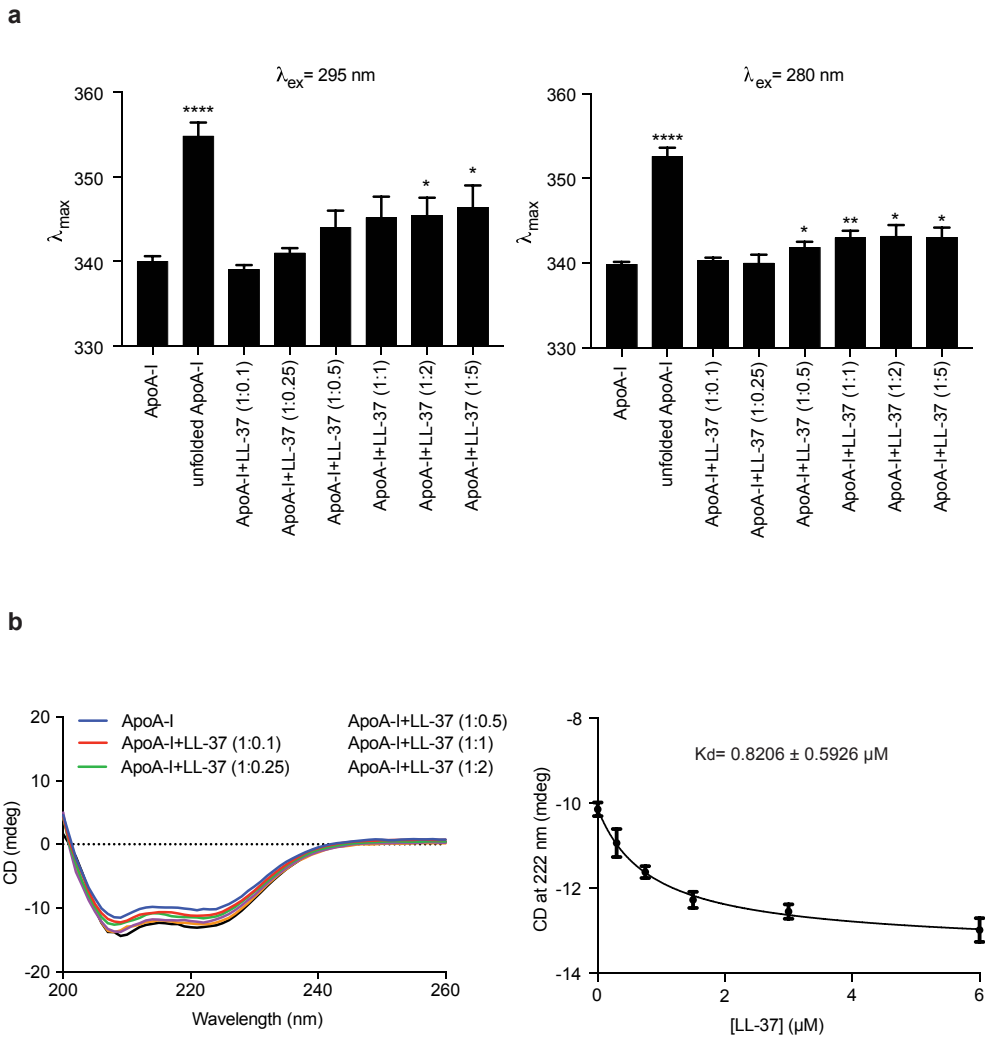

Fig. 1. ApoA-I is able to bind LL-37. 3 μM ApoA-I was incubated with increasing concentrations of LL-37 (protein to peptide molar ratios as indicated in the figure) in PBS for 30 min. At the end of incubation, intrinsic fluorescence (**a**) or CD spectra (**b**) of LL-37-bound ApoA-I were recorded. (**a**) Following excitation at 295 nm (left panel) or 280 nm (right panel), emission spectra were recorded and the maximum emission fluorescence (λ_{max}) values were plotted as a function of ApoA-I to LL-37 molar ratio. Unfolded ApoA-I (3 μM protein in 3 M Gnd-HCl) was used as a control. (**b**) CD spectra were recorded for each protein to peptide molar ratio (**left panel**) and the CD signal at 222 nm was plotted as a function of LL-37 concentration (**right panel**). The experimental data were fitted and K_d estimated by using one-site binding equation of non-linear regression, in GraphPad Prism 7. Data are the means ± SEM of five (**a**) or four (**b**) independent experiments. Significance is calculated according to *t*-test (*p < 0.05, **p < 0.01, ****p < 0.0001).

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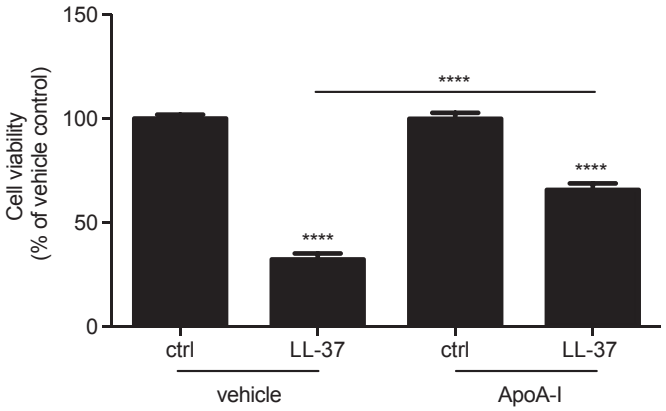

Fig. 2. ApoA-I rescues endothelial cells from LL-37 induced cytotoxicity. HUVEC were treated with LL-37 (6 μM) for 3 h, in the presence of absence of equimolar amount of ApoA-I. At the end of incubation, MTT assay was performed and cell viability calculated. Data are the means ± SEM of three independent experiments. Significance is calculated according to *t*-test (**p < 0.01, ****p < 0.0001).

3.2. ApoA-I protects against LL-37-induced cytotoxicity

We next wished to investigate whether ApoA-I protects endothelial cells against LL-37-induced cytotoxicity. HUVEC were treated with LL-37 in the presence or absence of wild-type ApoA-I at a 1:1 molar ratio. Treatment with 6 μM LL-37 reduced cell viability by about 60% (Fig. 2). Interestingly, as shown in Fig. 2, ApoA-I (3 μM) was able to attenuate the LL-37-induced cytotoxic effect by about 50%. Based on the 1:1 binding stoichiometry, a higher ApoA-I scavenging effect could possibly have been expected. However, the lack of full reversal of the LL-37-induced cytotoxicity by ApoA-I may be due to structural properties of the peptide. This notion is supported by the fact that LL-37 is, at physiological conditions, in an equilibrium between unstructured peptide and an α-helical conformation which tends to oligomerize [22]. Indeed, the binding of LL-37 by ApoA-I was previously reported to require α-helical conformation [13] suggesting a selective scavenging by ApoA-I where unstructured LL-37 peptide is left unaffected. This fraction of the LL-37 pool constitutes around 75% of the molecules in PBS, but presumably less under physiological conditions [22,23], suggesting that the protective ApoA-I scavenging effect may be

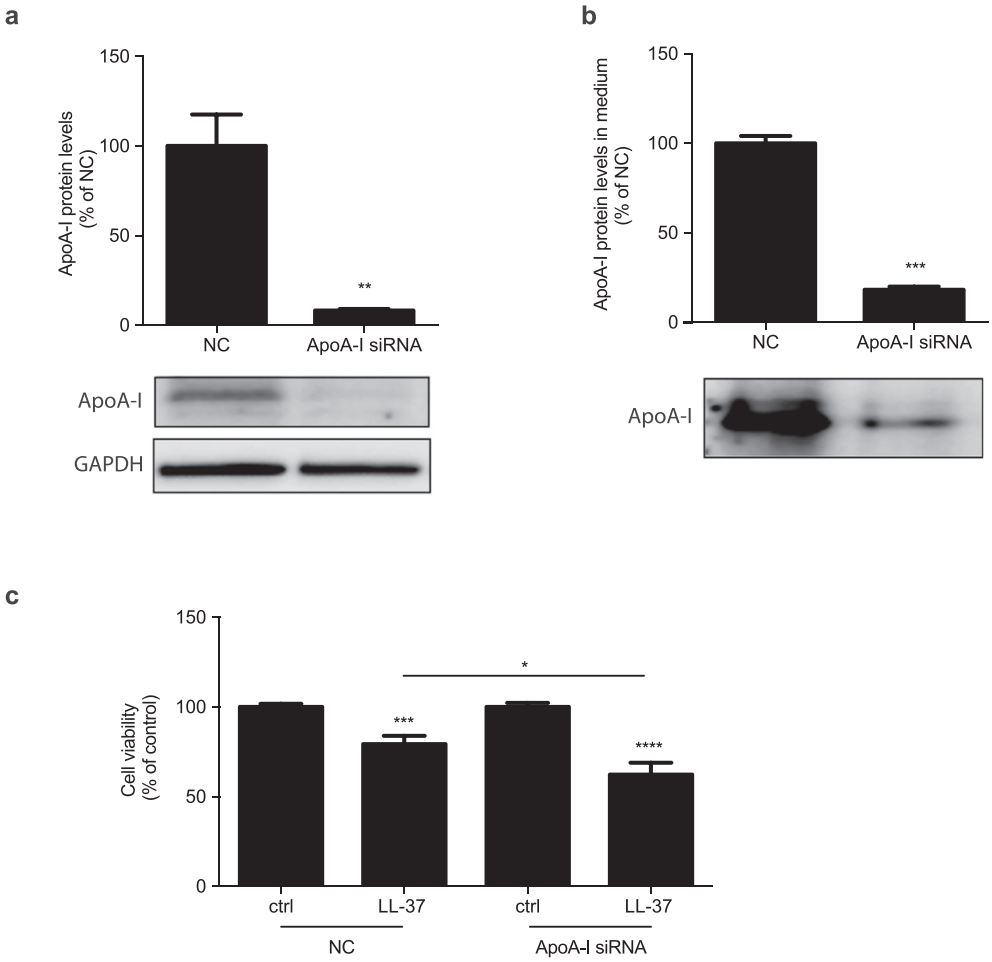

Fig. 3. Knockdown of endogenous ApoA-I sensitizes cells to LL-37-induced cytotoxicity. HepG2 cells were transfected with ApoA-I siRNA for 96 h. At the end of incubation, cell lysates and conditioned medium were analysed by western blotting using antibodies against ApoA-I (**a, b**). (**a**) Cell-associated ApoA-I was analysed from 30 μg of total cell proteins. ApoA-I signal was normalized to GAPDH. (**b**) Secreted ApoA-I levels were analysed from 20 μl of conditioned medium. (**c**) Cells treated with siRNA for ApoA-I and scramble transfected (NC, negative control) cells were incubated with LL-37 (12 μM) for 3 h and, at the end of incubation, the MTT assay was performed to determine cell viability. Data shown are the means ± SEM of three independent experiments. Significance is calculated according to *t*-test (**p < 0.01, ***p < 0.001).

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even more substantial *in vivo*. Interestingly, conformational changes of LL-37 affects is properties; the unstructured peptide is favorable when it comes to interactions with bacterial membranes [1,23] while certain effects on host cells are derived exclusively from α-helical peptide [22], further adding to the regulatory role of ApoA-I-to-LL-37 binding. This apparent regulated interaction with ApoA-I via conformational switching of the LL-37 may also be a reason for varied reports [10,12] on the LL-37 activity in the presence of ApoA-I.

In addition to wild-type ApoA-I, several variants have been reported to affect lipid metabolism. Two such variants of ApoA-I are associated either with an increased risk of cardiovascular disease (A164S variant) [24] or with a protection against cardiovascular disease (ApoA-I Milano) [25]. We therefore analysed their ability to attenuate the negative effects of the LL-37 peptide on human endothelial cell viability. However, as is shown in Fig. S2, both A164S and Milano variants exhibited the same degree of protection against LL-37-induced cytotoxicity in HUVEC as the wild type protein. Thus, the differences in atheroprotection of the two ApoA-I variants appear to be unrelated to their protection against LL-37-evoked cytotoxicity in endothelial cells.

3.3. Knockdown of ApoA-I by siRNA increases LL-37-induced cytotoxicity

To demonstrate that endogenously produced ApoA-I protects against LL-37-evoked cytotoxicity, we employed an siRNA approach to downregulate the protein expression in an ApoA-I producing cell type, HepG2 cells. As shown in Fig. 2 a and b, treatment with ApoA-I siRNA attenuated the protein levels by 80–90% in both the cell lysate and in the culture medium. In line with the ability of HepG2 cells to produce ApoA-I, this cell line appeared to be far less sensitive to LL-37-induced cytotoxicity as compared to HUVEC (Figs. 2 and 3c). However, the siRNA-induced reduction in ApoA-I expression was associated with a significant increase in the sensitivity against the peptide (Fig. 3c).

3.4. LL-37 binding to ApoA-I does not affect protein stability

In order to inspect the impact of LL-37 binding on ApoA-I stability, thermal melting of ApoA-I/LL-37 complex was assayed by both intrinsic fluorescence and CD spectroscopy (Fig. 4). The λ_{max} and the CD signal obtained at lower temperatures differed between the complex and the protein alone, confirming a conformational rearrangement of ApoA-I in the presence of LL-37. It is worth to notice that close to the physiological temperature (37 °C), the ApoA-I/LL-37 complex was still stable. The difference between the complex and ApoA-I only was abolished only at temperatures higher than 50 °C, probably due to the release of the LL-37 peptide from ApoA-I. Accordingly, the ApoA-I/LL-37 complex showed a Tm that was not significantly different from the one calculated for the protein itself, suggesting that LL-37 binding to ApoA-I does not affect protein stability.

In conclusion, we demonstrate that ApoA-I effectively binds LL-37 (K_d = 0.82 ± 0.59 μM) and that this interaction may represent the basis for the ApoA-I-evoked attenuation of LL-37-induced host cell cytotoxicity. ApoA-I, indeed, was able to rescue endothelial cells from LL-37 treatment while siRNA treatment of ApoA-I expressing HepG2 cells sensitized cells to LL-37-induced cytotoxicity. This mechanism is likely a part of ApoA-I's repertoire of atheroprotective functions. The binding of LL-37 to the ApoA-I protein resulted in a structural rearrangement of ApoA-I, but it did not result in a lower protein thermal stability, suggesting that LL-37 may not impair other functions of ApoA-I.

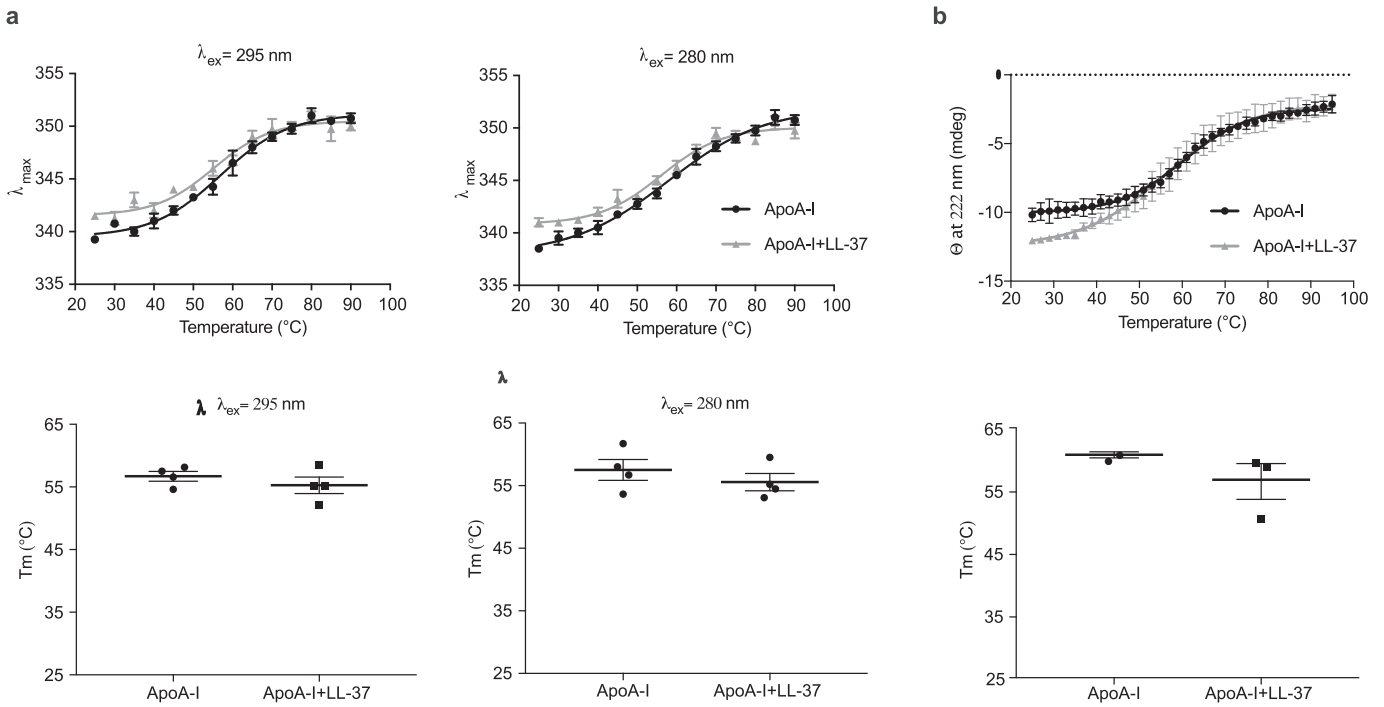

Fig. 4. LL-37 binding does not affect ApoA-I stability. ApoA-I protein (3 μM) was incubated with LL-37 (1:2 molar ratio) in PBS for 30 min and thermal unfolding of LL-37-bound ApoA-I was followed by intrinsic fluorescence (**a**) and CD spectroscopy (**b**). (**a**) Following excitation at either 295 nm (**left panel**) or 280 nm (**right panel**), emission spectra were recorded and curves were obtained by reporting the maximum emission fluorescence (λ_{max}) as a function of temperature. (**b**)The CD signal at 222 nm was recorded and plotted as a function of temperature. The experimental data were fitted and melting temperature (Tm) estimated (**a-b, lower panels**) by sigmoidal fitting using GraphPad Prism 7. Data shown are the means ± SEM.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2017.09.072.

Transparency document

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