A Photo-Activatable Peptide Mimicking Functions of Apolipoprotein A-I

Haruka Kawahara, Naoki Miyashita, Koki Tachibana, Yusuke Tsuda, Kyohei Morimoto, Kohei Tsuji, Akira Shigenaga, Akira Otaka, Tatsuhiro Ishida, and Keiichiro Okuhira*

Institute of Biomedical Sciences, Tokushima University Graduate School; 1–78–1 Sho-machi, Tokushima 770–8505, Japan.

Received February 10, 2019; accepted March 10, 2019

INTRODUCTION

Apolipoprotein A-I (apoA-I) plays a critical role in high-density lipoprotein (HDL) biogenesis, function and structural dynamics. Peptides that mimic apoA-I have a short amphipathic α-helical structure that can functionally recapitulate many of the same biologic properties of full-length apoA-I in HDL. Hence, they might be expected to have clinical applications in the reduction of atherosclerosis. However, nonspecific cellular efflux of cholesterol induced by apoA-I mimetic peptides might cause side effects that are, as yet, unidentified. In this study, we developed a photo-activatable peptide, 2F*, which is an 18 amino acid peptide mimicking apoA-I bearing an internal photocleavable caging group that is designed to assume an α-helical structure in response to a light stimulus and trigger efflux of cholesterol from cells. Without light irradiation, 2F* peptide showed a low tendency for the formation of α-helices, and therefore did not associate with lipids and failed to induce efflux of cholesterol. In addition, 2F* did not cause hemolysis under our experimental condition. Mass spectrometry indicated that, after light exposure, the caging group detached from 2F* and it assumed the α-helical structure in the presence of lipids, and enhanced cholesterol efflux from cells. Photo-activatable peptides such as 2F* that control cholesterol efflux following light stimulus may be useful for future atherosclerosis-reducing therapies.

Key words apolipoprotein; high-density lipoprotein (HDL); atherosclerosis; photocleavable peptide; cholesterol efflux

MATERIALS AND METHODS

Materials Dichloromethane, Dimethylformamide, Piperidine, Trifluoroacetic acid (TFA), Acetonitrile (MeCN), Diethyl ether, m-cresol, Thioanisole, 1,2,3-benzotriazol-1-ol monohydrate and 1,2-ethanediol were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). N,N′-Disopropylcarbodiimide was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). All 9-fluorenylmethoxycarbonyl (Fmoc) protected amino acids were purchased from CSBio, Inc. (Menlo Park, CA, U.S.A.). CLEAR-amide resin was purchased from Peptide Institute, Inc. (Osaka, Japan). 1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was purchased from Sigma-Aldrich (Merck Millipore, Burlington, MA, U.S.A.).

Peptide Synthesis and Light Activation Peptide 2F (Ac-DWLKAFYDKVAEKLKEAF-NH₂) and 2F*
(Ac-DWLKAFYDKV(DMNB)EKLKEAF-NH₂) were synthesized on CLEAR-Amide resin by a standard Fmoc solid-phase procedure. As building blocks, a Fmoc amino acid or dipeptide alanine-glutamic acid (Ala-Glu) bearing a 4,5-dimethoxy-2-nitrobenzyl caging group (Ala-(DMNB)Glu), prepared according to the literature, was employed. The peptide was purified to greater than 99% purity by reverse-phase HPLC, as assessed by electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS) (2F* MS data in Fig. S1). For analytical HPLC, reaction proceedings were monitored with reverse phased HPLC with C-18 column (Nacalai Tesque, Cosmosil 5C18-AR-II, 4.6 × 250 mm) by a linear gradient of 5–60% MeCN in 0.1% TFA buffer over 30 min at a flow rate of 1.0 mL/min, and monitored by UV detection at 220 nm (HITACHI Pump L-2130 with UV Detector L-2400). For preparative HPLC, the product was monitored with reverse phased HPLC with C-18 column (Nacalai Tesque, Cosmosil 5C18-AR-II, 20 × 250 mm) by a linear gradient of 35–50% MeCN for 2F or 30–45% MeCN for 2F* in 0.1% TFA buffer over 30 min at a flow rate of 10.0 mL/min, and monitored by UV detection at 220 nm. The peptides were solubilized in ultrapure water, then diluted to the desired concentration for experimental use. Photolysis was performed at room temperature using a Moritex MUV-202U with the filtered output (> 365 nm) of a 3000 mW/cm² Hg-Xe lamp. The photo-uncaging reaction of 2F* was monitored by analytical HPLC (Fig. 1B).

**Solubilization of 1,2-DMPC Vesicles by Peptides**

DMPC vesicles were formed as described previously. Briefly, weighed amounts of lipids were dissolved in chloroform-methanol (2:1 v/v). The organic solvent was removed by rotovaporation and the lipid film was hydrated with phosphate buffered saline (PBS) above its melting temperature of 24°C. Large unilamellar vesicles (LUV) were prepared by extruding a lipid suspension through a 200 nm polycarbonate filter yielding vesicles with the same approximate diameter. DMPC concentrations were determined with the choline oxidase-DAOS method (phospholipids C-test Wako kit). Peptides were incubated at 25°C for 30 min with DMPC vesicles (0.2 mg/mL) in PBS at a final peptide concentration of 50 µg/mL with continuous mixing and monitoring the absorbance at 325 nm in a Hitachi spectrophotometer U-3900H. Change in turbidity of samples was expressed as fold-change relative to time 0.

**Circular Dichroism (CD) Spectroscopy**

Far-UV CD spectra were recorded from 200 to 260 nm at 25°C using a Jasco J-1500 spectropolarimeter. The peptide solutions (50 µg/mL) in PBS or the peptides associated with egg phosphatidylcholine liposomes (egg PC; 200 µg/mL) were analyzed by circular dichroism spectroscopy. The α-helical content was determined from the molar ellipticity at 222 nm ([θ]_222) using the equation: % α-helix = [([θ]_222 + 3000)/(36000 + 3000)] × 100. Cholesterol efflux assay was performed as described previously. Briefly, Baby Hamster Kidney (BHK) stably transfected cells expressing a mifepristone-inducible human ABCA1 cDNA were incubated with apoA-I in Dulbecco’s modified Eagle’s medium (DMEM) or HBSS containing 0.02% bovine serum albumin (BSA) for 6 h. The cholesterol content in the supernatant was determined using enzymatic assay after lipid extraction from the supernatant.

**Hemolysis Assay**

Peptide solutions with different concentrations were mixed with 2% rat blood in PBS and then incubated for 24 h at 37°C. The samples were centrifuged at 750 × g for 5 min, and the hemolysis was assessed as a function of hemoglobin leakage by measuring the absorbance of supernatant at 540 nm. The percentage of hemolysis was determined thus:

\[
\% \text{hemolysis} = 100 \times \frac{\text{OD}_{540}[\text{test sample}] - \text{OD}_{540}[0 \% \text{hemolysis}]}{\text{OD}_{540}[100 \% \text{hemolysis}] - \text{OD}_{540}[0 \% \text{hemolysis}]} 
\]

To obtain 0 or 100% hemolysis, respectively, bloods were incubated either in PBS only or were mixed with distilled...
Statistics

Statistical analysis was performed using one-way ANOVA followed by the Bonferroni test. Results were regarded as significant for \( p < 0.05 \).

RESULTS

For the synthesis of the photoresponsive peptide called 2F*, the photocleavable 4,5-dimethoxy-2-nitrobenzyl (DMNB) group was inserted at the glutamic acid in the peptide 2F. The schematic for synthesized peptide derived by UV-radiation is illustrated in Fig. 1A. The photoreactivity of the peptide 2F* was confirmed as follows. The peptide was irradiated with UV light (>365 nm) for 5 min or 30 min. The reaction progress was monitored by HPLC (Fig. 1B), and the ESI-MS analysis confirmed that the resulting peptides had the exact same molecular mass as 2F. UV irradiation for 30 min caused almost complete cleavage of the DMNB group from 2F*, resulting in the generation of peptide 2F.

The ability of peptides derived from 2F* to solubilize lipids following UV irradiation was studied by adding peptides to DMPC vesicles, and following the decrease in turbidity as the peptides reorganize the vesicles into smaller structures. Within 10 min, the original 2F peptide decreased the relative turbidity, confirming that 2F could solubilize DMPC vesicles, consistent with a previous study. The activity of the 2F peptide was not influenced by UV-irradiation (Fig. 2A). In the absence of UV-irradiation, peptide 2F* did not induce any lipid solubilization, indicating that the presence of the DMNB group in the peptide successfully inhibited the association of the peptide with lipid. The UV-irradiation decreased the relative turbidity of DMPC vesicles by 80% within 10 min.

To determine the secondary structure of the derived peptides, far-UV circular dichroism spectroscopy was utilized (Fig. 2B). The peptide 2F, in the presence of lipids, shows two negative bands at 222 nm and 208 nm in the CD spectrum, typical of an \( \alpha \)-helix. Judging by the mean residue ellipticity at 222 nm, the original peptide 2F underwent a large increase in helicity from 7% in the lipid-free state to 63% helicity after lipid reconstitution (Table 1), similar to a previous report. The UV-irradiation did not affect the CD spectra of the 2F peptide.
indicating that the irradiation did not cause modification or disruption of peptide structures. In the absence of UV-irradiation, peptide 2F* showed a low degree of helicity (18.1%) in the presence of DMPC vesicles. The UV-irradiation of 2F* peptides caused a significant increase in helicity (50.1%). Taken together, these results indicate that UV-irradiation triggered formation of peptide 2F from peptide 2F* by enhancing cleavage of the DMNB group. As a consequence, 2F* peptides acquired the ability to solubilize DMPC vesicles by formation of α-helices promoting peptide-lipid association.

Next, we examined whether 2F* peptides promote cholesterol efflux from cells in the presence of UV-irradiation. The peptide was incubated with BHK cells expressing mifepristone and ABCA1 under different conditions, without and with mifepristone. Without UV-irradiation, regardless of the presence or absence of mifepristone, the peptide 2F* did not induce cholesterol efflux over a range of concentrations (10–50 µg/mL) (Fig. 3A). Following UV-irradiation, the 2F* peptide promoted the cholesterol efflux to a comparable level with the original 2F peptide in the presence of mifepristone (Fig. 3B). These results indicate that UV-irradiation of 2F* peptides mediated specific cholesterol efflux in an ABCA1-dependent manner.

Toxicity analysis of 2F peptides showed that they increased red blood cell hemolysis in a dose-dependent manner. Hemolysis caused by newly synthesized 2F* peptides showed that hemolysis was increased by UV irradiation in a dose dependent manner. Almost 20% of red blood cells were lysed at 100 µg/mL (Fig. 4) in 24 h at a temperature of 37°C, compared to a low hemolytic activity (less than 5%) in the absence of UV-irradiation (Fig. 4).

DISCUSSION

The most important structural protein in HDL, ApoA-I, consists of 243 amino acids with 10 amphipathic α-helices that are crucial for its efficient interaction with lipids. Recently, there has been increasing interest in the application of peptides that mimic the amphipathic helices in apoA-I as therapeutic agents.9,10 In this study, we developed a novel photoactivatable peptide mimicking apoA-I, programmed to induce lipid solubilization by UV-irradiation triggered by α-helix formation. Peptide 2F* was designed to contain a photocleavable caged glutamate that inhibits the hydrogen bonding required for the formation of α-helices (Fig. 1A). Peptide 2F* showed a low helix content in the presence of lipid vesicles (Fig. 2B, Table 1), no lipid solubilizing activity (Fig. 2A), very little hemolysis activity (Fig. 4), and no cholesterol efflux activity from cells (Figs. 3A, 3B). Of significance, following UV-irradiation, peptide 2F* showed a high helical content (Fig. 2B), significant lipid solubilizing activity (Fig. 2A), high hemolytic activity (Fig. 4) and cholesterol efflux activity (Fig. 3B), all consistent with the original 2F peptide. These results confirm that the 2F* peptide acquired the original activity of 2F peptide by liberating the DMNB group photolytically via UV-irradiation.

A series of apoA-I-mimetic peptides was designed to recapitulate the various in vivo beneficial function of HDL including reverse cholesterol transport activity, anti-inflammation, anti-oxidation and anti-proliferation,23–26 and some of these peptides have already shown a significant level of anti-atherogenicity in preclinical settings.27,28 However, some apoA-I-mimetic peptides have been reported to be hemolytic by nonspecific enhancement of cellular cholesterol efflux from red blood cells due to their high lipid solubilizing activity.13,14 Peptide 2F*, at higher concentrations, caused very little hemolysis unless activated by UV irradiation, while the original 2F peptide caused considerable hemolysis (Fig. 4). This suggests that the 2F* peptide is safe for intravenous administration compared to the original 2F peptide. It is possible that we might control not only cholesterol efflux at sites of atherosclerotic lesions, but systemic peptide-induced hemolysis by light stimulus under physiological conditions, which may provide advantages over the current apoA-I mimic peptides.

ApoA-I or its mimetic peptides induce the efflux of cholesterol and phospholipids from cells in both an ABCA1-dependent and -independent manner. ABCA1-mediated cholesterol efflux begins with the direct interaction of apoA-I or helix-type peptides to the cell membrane surface of ABCA1, and apoA-I or its peptide recruit cellular phospholipid and cholesterol translocated by ABCA1 to assemble HDL particles.29 ABCA1-independent cholesterol efflux involves a non-specific diffusion of lipids from the cell surface, in which cholesterol is trapped by various extracellular acceptors.

Several apoA-I mimetic peptides induce a non-specific cholesterol efflux, which leads hemolysis by associating with the surface of red blood cells and extracting lipids in an ABCA1-independent manner. A considerable hemolytic property has been reported about the peptide of 37 pA which linked two 2F with proline (2F-Pro-2F).13 Additionally systemic administration of apoA-I mimetic peptides may be saturated with lipids provided from plasma lipoproteins or surrounding cells before reaching the target site.13 Therefore, peptides that control the cholesterol efflux with external stimulus may be useful both for minimizing the risk of toxicity and for maximizing the efficacy.

Photoactivation of caged compounds enables the spatial
and temporal control of biomolecules in living cells or tissues. Such light-triggered systems, however, still has technical difficulties in in vivo use due to limited light penetration into the body. One strategy to overcome this limitation is to utilize the two-photon absorption process, where photosensitive groups simultaneously absorb two photons of near-infrared (NIR) light that can show relatively high levels of penetration to achieve a photochemical reaction similar to absorbing one photon of UV light.30) Interestingly, upconverting nanoparticles (UCNP) that convert from NIR to UV or visible light by virtue of two-photon absorption process is under investigation; this can trigger the photoreactions of adjacent photosensitive reagents.31) The combination of these materials with photosensitive peptides may lead to the efficient induction of the photochemical process in deep tissues, which expand the utility of our approach for future therapeutic use.

In conclusion, we have developed a new class of apoA-I-mimetic peptides and showed their superior physicochemical properties and biological activities in vitro. Through further studies on 2F* and improvements to prove its effectiveness in animal models, proof-of-concept results will be obtained for a novel approach for treatment of atherosclerosis.

Acknowledgments The authors are grateful to Dr. Theresa M. Allen for her helpful advice in developing the English manuscript. This study was supported, in part, by a Grant-in-Aid for Scientific Research (25430164, 16K08236 and 16KK0203) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by the Nagai Foundation, Tokyo, by Takahashi Industrial and Economic Research Foundation, Tokyo, and by a research program for development of intelligent Tokushima artificial exosome (tEX) from Tokushima University.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

REFERENCES

1) Gordon T, Castelli WP, Hjortland MC, Kannel WB, Dawber TR. High density lipoprotein as a protective factor against coronary heart disease. The Framingham study. Am. J. Med., 62, 707–714 (1977).
2) Assman G, Schulte H, Von Eckardstein A, Huang Y. High-density lipoprotein cholesterol as a predictor of coronary heart disease risk. The PROCAM experience and pathophysiological implications for reverse cholesterol transport. Atherosclerosis, 124 (Suppl.), S11–S20 (1996).
3) Ansell BJ, Navab M, Hama S, Kamranpour N, Fonarow G, Hough G, Rahmani S, Mottahehed H, Dave R, Reddy ST, Fogelman AM. Inflammatory/antiinflammatory properties of high-density lipoprotein distinguish patients from control subjects better than high-density lipoprotein cholesterol levels and are favorably affected by simvastatin treatment. Circulation, 108, 2751–2756 (2003).
4) Navab M, Hama SY, Hough GP, Subbanagounder G, Reddy ST, Fogelman AM. A cell-free assay for detecting HDL that is dysfunctional in preventing the formation of or inactivating oxidized phospholipids. J. Lipid Res., 42, 1308–1317 (2001).
5) Suc I, Escargueil-Blanc I, Troy I, Salvayre R, Negre-Salvayre A. HDL and apoA prevent cell death of endothelial cells induced by oxidized LDL. Arterioscler. Thromb. Vasc. Biol., 17, 2158–2166 (1997).
6) Chen LY, Mehta JL. Inhibitory effect of high-density lipoprotein on platelet function is mediated by increase in nitric oxide synthase activity in platelets. Life Sci., 55, 1815–1821 (1994).
7) Badimon JJ, Badimon L, Fuster V. Regression of atherosclerotic lesions by high density lipoprotein plasma fraction in the cholesterol-fed rabbit. J. Clin. Invest., 85, 1234–1241 (1990).
8) Miyazaki A, Sakuma S, Morikawa W, Takie T, Mika E, Terano T, Sakai M, Hakamata H, Sakamoto YI, Naito M, Ruan Y, Takahashi K, Ohta T, Horiuchi S. Intravenous injection of rabbit apolipoprotein A-I inhibits the progression of atherosclerosis in cholesterol-fed rabbits. Arterioscler. Thromb. Vasc. Biol., 15, 1882–1888 (1995).
9) Recio C, Maione F, Igbaal AJ, Mascolo N, De Feo V. The potential therapeutic application of peptides and peptidomimetics in cardiovascular disease. Front. Pharmacol., 7, 52 (2016).
10) Stoekenbroek RM, Stroes ES, Hovingh GK. ApoA-I mimetics. Handb. Exp. Pharmacol., 224, 631–648 (2015).
11) Lund-Katz S, Phillips MC. High density lipoprotein structure-function and role in reverse cholesterol transport. Subcell. Biochem., 51, 183–227 (2010).
12) Adorni MP, Zinetti F, Billheimer JT, Wang N, Rader DJ, Phillips MC, Rothblat GH. The roles of different pathways in the release of cholesterol from macrophages. J. Lipid Res., 48, 2453–2462 (2007).
13) Sethi A, Stonik JA, Thomas F, Demosky SJ, Amar M, Neufeld E, Brewer HB, Davidson WS, D’Souza W, Sviridov D, Remaley AT. Asymmetry in the lipid affinity of bivalent amphipathic peptides: a structural determinant for the specificity of ABCA1-dependent cholesterol efflux by peptides. J. Biol. Chem., 283, 32273–32282 (2008).
14) D’Souza W, Stonik JA, Murphy A, Demosky SJ, Sethi AA, Moore XL, Chin-Dusting J, Remaley AT, Sviridov D. Structure/function relationships of apolipoprotein A-I mimetic peptides: implications for antiatherogenic activities of high-density lipoprotein. Circ. Res., 107, 217–227 (2010).
15) Conroy I, Jolliffe RA, Payne RJ. Synthesis of N-linked glycopeptides via solid-phase aspartylation. Org. Biomol. Chem., 8, 3723–3733 (2010).
16) Segall ML, Dhanasekaran P, Baldwin F, Anantharamaiah GM, Weisgraber KH, Phillips MC, Lund-Katz S. Influence of apoE domain structure and polymorphism on the kinetics of phospholipid vesicle solubilization. J. Lipid Res., 43, 1688–1700 (2002).
17) Nagao K, Hata M, Tanaka K, Takechi Y, Nguyen D, Dhanasekaran P, Lund-Katz S, Phillips MC, Saito H. The roles of C-terminal helices of human apolipoprotein A-I in formation of high-density lipoprotein particles. Biochim. Biophys. Acta - Mol. Cell Biol. Lipids, 1811, 80–87 (2014).
18) Sparks DL, Lund-Katz S, Phillips MC. The charge and structural stability of apolipoprotein A-I in discoidal and spherical reconstituted high density lipoprotein particles. J. Biol. Chem., 267, 25839–25847 (1992).
19) Okuhira K, Tsujita M, Yamauchi Y, Abe-Dohmae S, Kato K, Handa J, Yokoyama S. Potential involvement of dissociated apoA-I in the ABCA1-dependent cellular lipid release by HDL. J. Lipid Res., 45, 645–652 (2004).
20) Vaughan AM, Oram JF. ABCA1 redistributes membrane cholesterol independent of apolipoprotein interactions. J. Lipid Res., 44, 1373–1380 (2003).
21) Venkatachalapathy YV, Phillips MC, Epand RM, Epand RF, Tytler EM, Segrest JP, Anantharamaiah GM. Effect of end group blockage on the properties of a class A amphipathic helical peptide. Proteins Struct. Funct. Bioinforma., 15, 349–359 (1993).
22) Egashira M, Gorbenko G, Tanaka M, Saito H, Molotkovsky J, Nakano M, Handa T. Cholesterol modulates interaction between an amphipathic class A peptide, Ac-I8A-NH2, and phosphatidylcholine bilayers. Biochemistry, 41, 4165–4172 (2002).
23) Bielicki JK, Zhang H, Cortez Y, Zheng Y, Narayanaswami V, Patel A, Johansson J, Azhar S. A new HDL mimetic peptide that stimulates cellular cholesterol efflux with high efficiency greatly reduces atherosclerosis in mice. *J. Lipid Res.*, 51, 1496–1503 (2010).

24) Amar MJA, D’Souza W, Turner S, Demosky S, Sviridov D, Stonik J, Luchoomun J, Voogt J, Hellerstein M, Sviridov D, Remaley AT. 5A apolipoprotein mimetic peptide promotes cholesterol efflux and reduces atherosclerosis in mice. *J. Pharmacol. Exp. Ther.*, 334, 634–641 (2010).

25) Tabet F, Remaley AT, Segaliny AI, Millet J, Yan L, Nakhaia S, Barter PJ, Rye KA, Lambert G. The 5A apolipoprotein A-I mimetic peptide displays antiinflammatory and antioxidant properties in vivo and in vitro. *Arterioscler. Thromb. Vasc. Biol.*, 30, 246–252 (2010).

26) Ganapathy E, Su F, Meriwether D, Devarajan A, Grijalva V, Gao F, Chattopadhyay A, Anantharamaiah GM, Navab M, Fogelman AM, Reddy ST, Farias-Eisner R, D-4F, an apoA-I mimetic peptide, inhibits proliferation and tumorigenicity of epithelial ovarian cancer cells by upregulating the antioxidant enzyme MnSOD. *Int. J. Cancer*, 130, 1071–1081 (2012).

27) Chattopadhyay A, Navab M, Hough G, Gao F, Meriwether D, Grijalva V, Springstead JR, Palgnachari MN, Namiri-Kalantari R, Su F, Van Lenten BJ, Wagner AC, Anantharamaiah GM, Farias-Eisner R, Reddy ST, Fogelman AM. A novel approach to oral apoA-I mimetic therapy. *J. Lipid Res.*, 54, 995–1010 (2013).

28) Iwata A, Miura S, Zhang B, Imaizumi S, Uehara Y, Shiomi M, Saku K. Antiatherogenic effects of newly developed apolipoprotein A-I mimetic peptide/phospholipid complexes against aortic plaque burden in Watanabe-heritable hyperlipidemic rabbits. *Atherosclerosis*, 218, 300–307 (2011).

29) Tang C, Vaughan AM, Anantharamaiah GM, Oram JF. Janus kinase 2 modulates the lipid-removing but not protein-stabilizing interactions of amphipathic helices with ABCA1. *J. Lipid Res.*, 47, 107–114 (2006).

30) Ellis-Davies GCR. Caged compounds: Photorelease technology for control of cellular chemistry and physiology. *Nat. Methods*, 4, 619–628 (2007).

31) Wu S, Butt HJ. Near-infrared-sensitive materials based on upconverting nanoparticles. *Adv. Mater.*, 28, 1208–1226 (2016).