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Properties of apolipoprotein E derived peptide modulate their lipid-binding capacity and influence their anti-inflammatory function ☆

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ABSTRACT

Apolipoprotein-derived peptides are promising candidates for the treatment of various inflammatory conditions. The beneficial effects of these peptides are based on multiple mechanisms; prominent among them being high-affinity binding to pro-inflammatory oxidized phospholipids (Ox-PLs) and facilitating their sequestration/metabolism/clearance in the body. This indicates that peptides which can bind exclusively to Ox-PLs without recognizing normal, non-oxidized phospholipids (non-Ox-PLs) will be more potent anti-inflammatory agent than that of the peptides that bind to both Ox-PLs and non-Ox-PLs. In order to develop such Ox-PL-specific peptides, the knowledge about the properties (molecular determinants) of peptides that govern their Ox-PL preference is a must. In this study we have synthesized eleven peptides corresponding to the conserved regions of human apolipoprotein E and compared their biochemical properties, lipid-binding specificities, and anti-inflammatory properties. Our results show that these peptides exhibit considerably different specificities towards non-Ox-PL and different species of Ox-PLs. Some of these peptides bind exclusively to the Ox-PLs and inhibit the pro-inflammatory function of Ox-PLs in human blood. Biochemical characterization revealed that the peptides possess substantially different properties. Our results suggest that physicochemical properties of peptides play an important role in their lipid-binding specificity.

1. Introduction

Apolipoprotein-derived peptides have emerged as potential candidates for the treatment of cardiovascular disorders and other chronic inflammatory conditions [1–3]. These peptides have been shown to reduce the development of atherosclerosis and other inflammatory conditions in a variety of animal models [4,5]. Recent studies have suggested that these peptides are safe and well tolerated in the humans [6,7]. The beneficial effects of these peptides are based on multiple mechanisms; prominent among them is their capacity to bind pathogenic Ox-PLs in the body and facilitating their sequestration/metabolism/clearance thereby inhibiting/reducing the deleterious effects of Ox-PLs in the body [1–5,8,9].

Increased oxidative stress is involved in the initiation and propagation of various disease conditions including atherosclerosis and other chronic inflammatory diseases [9,10]. Free radical or enzyme-mediated oxidation of lipids causes a variety of changes in the lipid molecules which include acyl chain truncation and/or modifications of the acyl chain by polar functional groups [11,12]. As a result of this, a large number of Ox-PL species are generated from a single type of non-Ox-PL. These different species of Ox-PLs differ considerably in their molecular structures, chemical compositions, and physicochemical as well as biological effects [11–13]. Ox-PL species interact with a variety of cells by binding to specific receptors and in general give rise to inflammatory signals. There is overwhelming evidence that in humans the level of circulating Ox-PLs increases in many chronic and acute inflammatory conditions [9,10]. Thus, decreasing the concentration of Ox-PLs in the body will have beneficial effects.

Literature suggests that non-Ox-PLs binding capacity of apolipoprotein A-I-derived peptides do not correlate with their anti-inflammatory properties and the peptides having high affinity towards Ox-PLs were found to be bioactive as anti-inflammatory agents [8,14,15]. A recent study of an apolipoprotein-derived peptide in humans with coronary heart disease (CHD) failed to show improvement in the anti-inflammatory markers in the subjects despite achieving sufficient level of the peptide in the plasma [7]. One of the reasons for this could have

Abbreviations: ApoE, apolipoprotein E; CD spectroscopy, circular dichroism spectroscopy; COX-2, cyclooxygenase-2; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; ELISA, enzyme-linked immunosorbent assay; IL-8, interleukin 8; KOdiA-PC, 1-palmitoyl-2-(5-keto-6-octendioyl)-*sn*-glycero-3-phosphocholine; LDL, low density lipoprotein; MCP-1, monocyte chemoattractant protein 1; MDA, malondialdehyde; MLV, multilamellar vesicle; non-Ox-PL, non-oxidized phospholipid; Ox-PLs, oxidized phospholipids; Ox-PAPC, oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; PAPC, 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine; PBS, phosphate buffer saline; PGPC, 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine; PL, phospholipid; POVPC, 1-palmitoyl-2-(5′-oxo-valeroyl)-*sn*-glycero-3-phosphocholine; qRT-PCR, quantitative real time polymerase chain reaction; RNA, ribonucleic acid; SDS, sodium dodecyl sulfate; TBARS, thiobarbituric acid reactive substances; TFE, trifluoroethanol; tOx-PLs, truncated oxidized phospholipids; VEGF, vascular endothelial growth factor

☆ Conflict of interest: None.

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been the high affinity of the peptide towards Ox-PLs as well as normal non-Ox-PLs in the body, which could have reduced/blocked the anti-inflammatory actions of the peptide. This means that the peptide that will bind exclusively to Ox-PLs without binding to the normal non-Ox-PLs would be more potent in exerting anti-inflammatory effects.

Human apolipoprotein E (apoE) is an exchangeable apolipoprotein present in the plasma and in the cerebrospinal fluid (CSF) and participates in the transport and metabolism of lipids [16]. Anti-atherosclerotic and anti-inflammatory roles of apoE have been experimentally demonstrated in several animal models and its normal level in human plasma is known to prevent the development of atherosclerotic plaque in the vasculature [16,17]. Structurally, apoE is composed of two independent domains: a 22-kDa, low density lipoprotein-receptor-binding N-terminal domain and a 10-kDa, lipid-binding C-terminal domain. Like other exchangeable apolipoproteins, apoE contains a number of conserved repeats of ~22-amino acids often separated by a proline residue [16,18]. These conserved repeats have propensity to form amphipathic α-helical structures, a motif responsible for the lipid-binding and biological properties of apolipoprotein-derived peptides. In apoE the distribution of 22-residue conserved repeats span between residues 29 and 288 and the punctuation by the proline residue between the repeats is less pronounced [16,18].

To see the effect of properties of the peptide on their lipid-binding preference and their biological function, we have synthesized 11 peptides corresponding to the conserved regions of human apoE and studied their biochemical properties, lipid-binding specificities, and anti-inflammatory properties. Our results show that these peptides possess considerably different lipid-binding specificities. Some of the peptides bind exclusively to Ox-PLs and inhibited the pro-inflammatory function of Ox-PLs in human blood. Biochemical characterization reveals that these peptides possess noticeably different properties. These results suggest that physicochemical properties of the peptides play an important role in their lipid-binding specificity.

2. Materials and methods

2.1. Materials

1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (PAPC) was purchased from Avanti Polar Lipids (Alabaster, AL). 1-palmitoyl-2-(5′-oxo-valeroyl)-*sn*-glycero-3-phosphocholine (POVPC), 1-palmitoyl-2-glutaroyl-*sn*-glycero-3-phosphocholine (PGPC), and 1-palmitoyl-2-(5-keto-6-octendioyl)-*sn*-glycero-3-phosphocholine (KOdiA-PC) were purchased from Cayman Chemical (Ann Arbor, MI). 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), chloroform, EDTA, trifluoroethanol (TFE), sodium dodecyl sulfate (SDS), sodium chloride, goat anti-rabbit IgG ALKA, BCIP, NBT, and the primers used for qRT-PCR experiments were purchased from Sigma-Aldrich (Bangalore, India). A NativeMark™ protein standard of known Stokes diameter was purchased from Invitrogen (Bangalore, India). RiboPure™-Blood Kit was purchased from Ambion (Bangalore, India). One-Step SYBR® Ex Taq™ qRT-PCR kit obtained from Takara Bio Inc. (Otsu, Japan) and human IL-8 ELISA kit was purchased from Krishgen BioSystems (Mumbai, India). All other reagents used were of analytical grade. Buffers used were prepared in double distilled deionized water.

2.2. Peptides

Peptides corresponding to the conserved amphipathic repeats of human apoE were custom synthesized by the Genscript Corp. (NJ, USA). The 4F peptide, made up of L-amino acids and used as a control apolipoprotein-derived peptide, was a kind gift from Dr. G. M. Anantharamaiah (Department of Medicine, University of Alabama at Birmingham, AL, USA). All the peptides were >95% pure as judged by the HPLC analysis and possessed the correct mass as determined by electrospray mass spectrometry. All the peptides were acetylated at the amino terminus and amide-capped at the carboxyl terminus. Removal of the residual TFE, generally present in trace amounts in purified peptide preparations, was done by the procedure reported previously [19]. The pure peptides (in powder form) were dissolved in phosphate buffered saline (PBS) containing 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, and pH 7.4 and the stock peptide solutions were aliquoted and stored at −20 °C. Concentration of the peptides was determined by BCA assay [20].

2.3. Preparation of Ox-PAPC

Ox-PAPC was prepared by the air-oxidation of PAPC by using the procedure previously reported [21–22].

2.4. Preparation of lipid vesicles and incubation of the peptides with lipid vesicles in buffer

Appropriate amount of lipid stock solution in chloroform (or in methanol) was taken in a tube and the organic solvent was removed under nitrogen followed by desiccation for ~3 h in vacuo. The dried lipid film was then hydrated by adding an appropriate amount of the PBS and vortexing the tubes at room temperature (25 °C) to form multilamellar vesicle (MLV)/lipid suspension. It is important to note that above its critical micellar concentration, DMPC and POPC form MLVs in aqueous solution while Ox-PLs used in this study (POVPC, PGPC, KOdiA-PC) do not form MLV but form 'micellar' aggregates in aqueous solution, the exact structural characteristics of which is not known [12]. To see the interaction of the peptides with lipids, the individual peptide was added to the measured volume of lipid suspension to obtain the desired peptide: lipid molar ratio. The final concentration of the peptide was 30 μM and the peptide: lipid molar ratio used were 1:0, 1:0.5, 1:1, 1:4, 1:7, 1:14, 1:20, and 1:30. The peptide: DMPC mixtures were incubated above and below the transition temperature of DMPC (24 °C) i.e. 10 min at 30 °C followed by 10 min at 20 °C. After 3 heating and cooling transition cycles, the mixtures were incubated overnight at 24 °C. The peptide: POPC (±Ox-PL) mixtures and the peptide: Ox-PL mixtures were incubated at room temperature (25 °C), as binding of the peptides to these lipids was found to be independent of the temperature of incubation.

2.5. Gel electrophoresis and Western blotting

Electrophoresis of the samples under non-denaturing conditions was carried out using the method of Laemmli [23]. Peptide: lipid binary mixtures or human plasma samples were individually mixed with the loading dye and electrophoresis was carried out in a mini VE electrophoresis unit (GE Healthcare, Uppsala, Sweden) under the condition of constant voltage. After electrophoresis, the gels were stained with silver stain. The Stokes diameter of the peptide: lipid complexes were determined from a calibration curve obtained by using standard protein markers. Formation of the pre-β HDL particles by the peptides was detected by Western blotting using anti-human apoA-I antibody as a primary antibody (a kind gift from Prof. Larry Swift, Department of Pathology, Vanderbilt University School of Medicine, Nashville, Tennessee) [24]. For Western blotting, the proteins on the gel were transferred onto the nitrocellulose membrane in transfer buffer using the following conditions: 50 V, 500 mA, and 2.5 h. The non-specific sites in the membranes were then blocked by using 5% BSA in Tris-buffered saline tween-20 (TBST) containing 10 mM Tris, pH 7.8, 150 mM NaCl, and 0.1% Tween 20. Subsequently, the membranes were washed with TBST, followed by incubation with polyclonal rabbit anti-human apoA-I antibody overnight at 4 °C. The membranes were then washed with TBST and were incubated with the secondary antibody (goat anti-rabbit IgG ALKA, Sigma) for 1 h. After washing in TBST, the bands were developed using BCIP-NBT reagent.