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Apolipoprotein E isoform-specific binding to the low-density lipoprotein receptor

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Abstract

Apolipoprotein E (apoE) is a ligand for members of the low-density lipoprotein receptor (LDLR) family and functions in plasma cholesterol homeostasis. A fluorescence-based assay has been employed in molecular studies of receptor-ligand interactions. Competition experiments revealed isoform specific differences in binding of lipid-associated apoE N terminal (NT) domain to a recombinant soluble LDLR (sLDLR). In a similar manner, lipid associated, but not lipid-free, full-length apoE3 showed binding activity to sLDLR. The molecular chaperone, Receptor Associated Protein, inhibited apoE3-NT-phospholipid complex binding to sLDLR. Kinetic studies of apoE3-NT-phospholipid complex interaction with sLDLR revealed time dependent effects of apoE-NT isoform binding to sLDLR. The results reveal a discerning method for study of the molecular basis of ligand interactions that likely influence receptor function in maintenance of whole body cholesterol homeostasis.

Introduction

Classical studies by Brown and Goldstein revealed that the low-density lipoprotein receptor (LDLR) pathway plays a key role in plasma cholesterol homeostasis [1]. Following discovery of the LDLR, a growing number of homologous receptors have been identified [2]. The prototype LDLR binds two physiologically important ligands, apolipoprotein B-100 and apolipoprotein E (apoE). Interestingly, apoE is recognized as a ligand for several members of the LDLR family and represents one of the best-characterized ligands in terms of its structural properties and physiological effects. ApoE contains 299 amino acids and is comprised of two structural domains, a 22 kDa N-terminal (NT) domain and a 10 kDa C-terminal domain [3]. In the absence of lipid the NT domain is organized as a four-helix bundle [4]. Each of the four helices are amphipathic and orient their non polar face toward the interior of the bundle, forming a leucine zipper type motif. Amino acid residues essential for interaction with the LDLR have been localized to the NT domain. In fact, the NT domain is both necessary and sufficient for full LDLR binding activity. Although lipid-free apoE is unable to bind LDLR, lipid association induces the protein to adopt a receptor active conformation [5,6].

Human apoE exists as one of three major isoforms, apoE2, apoE3 and apoE4. The isoforms differ at amino acid positions 112 and 158. ApoE3, the most common isoform, contains cysteine at position 112 and arginine at position 158. ApoE2 has cysteine at both these positions whereas apoE4 has arginine. In cell culture based binding assays, it has been shown apoE

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isoforms show different levels of affinity for LDLR [6]. ApoE3 and apoE4 bind to LDLR with high affinity while apoE2 binding is much weaker [7]. Isoform (allele)-specific effects include the association of apoE2 with the genetic disorder type III hyperlipoproteinemia [8] and association of apoE4 with increased risk for both atherosclerosis and Alzheimer's disease [9]. Whereas existing cell based assays reveal isoform specific differences in apoE binding that correlate with various maladies, they are not well suited to studies of molecular mechanism. In an effort to circumvent problems intrinsic to whole cell studies, we have employed a solution assay in which binding is monitored by changes in inter-molecular fluorescence resonance energy transfer (FRET) between receptor and ligand [10]. In the present study, isoform specific binding of apoE to a recombinant soluble LDLR (sLDLR) and the effect of the molecular chaperone, Receptor Associated Protein (RAP) [11] on apoE receptor binding are described.

Material and methods

Analytical procedures

Protein concentrations were determined by the BCA assay (Pierce Chemical Co., Rockford, IL, USA) using bovine serum albumin as a standard. SDS-PAGE was performed on 4–20 % acrylamide gradient slab gels electrophoresed at 35 mA constant current. Native-PAGE was performed on 4–20 % acrylamide slab gels run at a constant 125 V for 12 h. Gels were stained with Gel Code (Pierce Chemical Co., Rockford, IL, USA) according manufacturer's instructions.

Protein expression/isolation

Recombinant human apoE-NT isoforms and Trp-null apoE3-NT were produced and isolated as described by Fisher et al. [12]. Briefly, apoE-NT isoforms were expressed in *E. coli* BL21 cells and isolated from the culture medium by heparin Sepharose chromatography and reversed phase high performance liquid chromatography. Recombinant full-length apoE3 was expressed in *E. coli* and isolated as described elsewhere [13]. Recombinant human apolipoprotein A-I (apoA-I) was prepared as described by Ryan et al. [14]. To obtain RAP, a pET15b plasmid harboring the coding sequence for RAP plus a N-terminal His-tag was transformed into *E. coli* BL21 cells. Following expression, RAP was isolated from a solubilized bacterial cell pellet by Ni²⁺ chelation chromatography and heparin Sepharose chromatography. sLDLR (N-terminal residues 1–699) was isolated from conditioned media of stably transfected HEK 293 cells as described by Yamamoto et al. [10].

Apolipoprotein-phospholipid complexes

1,2-Dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was purchased from Avanti Polar Lipids (Pelham, AL, USA). DMPC was dissolved in chloroform:methanol (3:1; v/v) and dried under a stream of N₂ gas as a thin film in a glass tube. Following dispersion of DMPC in 50 mM sodium phosphate, pH 7.0, 150 mM NaCl, apolipoproteins were added and the sample bath sonicated at 24 °C until clear [15].

sLDLR binding assay

Two µg sLDLR (in 20 mM Tris, pH 7.2, 2 mM CaCl₂, 90 mM NaCl) was incubated with 1 µg Trp-deficient apoE3-NT previously labeled on Cys112 with the fluorescent probe, N-(iodoacetyl)-N'-(5-sulfo-1-naphthyl) ethylenediamine (AEDANS) and complexed with DMPC [15]. Interaction between AEDANS-Trp-null apoE3-NT•DMPC and sLDLR is detected by FRET between excited Trp residues in sLDLR and the AEDANS moiety covalently attached to Trp-null apoE3-NT•DMPC [10]. Unlabeled competitor ligands (specified apoE-NT isoform•DMPC complexes or RAP) were incubated with sLDLR and AEDANS-Trp null apoE-NT•DMPC at 25°C for 10 min (final sample volume 300 µl). Following incubation, samples

were excited at 280 nm and fluorescence emission intensity at 470 nm determined (slit width 5.0 nm) on a Perkin-Elmer LS50b luminescence spectrometer.

Results and Discussion

ApoE-NT•DMPC disk particle formation

Isolated recombinant apoE-NT isoforms gave rise to a single major band on SDS-PAGE (Figure 1). In addition, complexing with DMPC resulted in formation of discrete lipid particles with diameters in the range of 18 nm. Based on previous studies, it is known that these particles exist as disk-shaped phospholipid bilayers wherein two or more apoE-NT molecules circumscribe the disk perimeter through interaction with phospholipid fatty acyl chains [16, 17].

Interaction of apoE-NT isoforms with sLDLR

To study isoform specific binding of apoE-NT to sLDLR, fluorescence-based competition binding assays were performed. The assay measures the ability of candidate ligands to compete with AEDANS labeled, Trp null apoE3-NT•DMPC disks for binding to sLDLR [10]. Lipid-free apoE2-NT was unable to interfere with FRET from excited Trp residues in sLDLR to the AEDANS moiety covalently bound to Cys112 of Trp null apoE3-NT•DMPC disks. Likewise, relatively poor binding was observed for apoE2-NT•DMPC disks (Figure 2, panel A). Whereas, lipid-free apoE3-NT and apoE4-NT were also poor competitors, apoE3-NT•DMPC disks and apoE4-NT•DMPC disks displayed concentration-dependent competition (Figure 2, panels B and C). In Figure 2, panel D different apoE-NT•DMPC and apoA-I•DMPC were directly compared. ApoE3-NT•DMPC and apoE4-NT•DMPC showed the strongest sLDLR binding activity while apoE2-NT•DMPC displayed weaker binding, although a small amount of competition was observed with this ligand. The negative control, human apoA-I•DMPC, showed no binding.

Full-length apoE3 and RAP binding to sLDLR

Full-length apoE3•DMPC manifested concentration-dependent binding to sLDLR (Figure 3) while lipid-free full-length apoE3 was a poor competitor. The affinity of full-length apoE3 for sLDLR was quite similar to that of apoE3-NT, consistent with the conclusion that the NT domain possesses all of the structural requirements for LDLR binding [7]. RAP is a chaperone found primarily in the endoplasmic reticulum that plays a role in folding and exocytic trafficking of members of the LDLR gene family, including megalin and the LDLR-related protein [18–20]. Using isolated recombinant RAP, competition-binding studies with sLDLR were performed (Figure 3, panel B). Unlike apoE, however, the binding interaction between RAP and sLDLR was not dependent on lipid association. This result shows that binding interactions with alternate, non apoE, LDLR binding proteins can be detected using the present FRET-based assay.

Studies of the time dependent sLDLR/apoE-NT isoforms binding

To examine time-dependent interactions between sLDLR and apoE-NT isoforms, sLDLR and specified unlabeled apoE-NT•DMPC were combined and incubated. After 0.5 min AEDANS-Trp null apoE3-NT•DMPC was added with monitoring of fluorescence emission at 470 nm (excitation 280 nm) as a function of time (Figure 4). The extent of enhancement in AEDANS emission, corresponding to displacement of unlabeled ligand from sLDLR, was dependent upon the competitor apoE-NT isoform employed. In the absence of unlabeled competitor ligand, addition of AEDANS-Trp null apoE3-NT•DMPC induced a rapid strong enhancement in fluorescence emission at 470 nm, consistent with interaction of this ligand with the receptor. When sLDLR was pre-incubated with various apoE-NT•DMPC, isoform specific differences

in AEDANS fluorescence intensity as a function of time were observed. Consistent with the results of competition binding assays in Figure 2, apoE2-NT•DMPC was more rapidly displaced from the receptor than either apoE3-NT•DMPC or apoE4-NT•DMPC. With the latter two competitor ligands, a gradual enhancement in AEDANS fluorescence emission was seen over the course of 5 min, consistent with relatively slow displacement of bound unlabeled ligand by the labeled competitor ligand.

In summary, members of LDLR family function in numerous processes including regulation of proteases and their inhibitors, protein re-absorption from glomerular filtrate, neuronal migration, signal transduction and intracellular sorting. In the present study, a FRET-based binding assay has been employed to investigate molecular details of apoE binding to sLDLR. The observed affinities of apoE-NT isoforms for sLDLR are consistent with earlier cell culture based assay results [21]. In contrast to other assays, however, the present studies were performed in a cell- and detergent-free solution using isolated buffer soluble components under conditions that can be easily manipulated. In addition, the amount of material consumed per assay is low although sensitivity is high. The ability to monitor receptor-ligand interactions in real time indicates this system is amenable to determination of association and dissociation kinetic parameters with the additional potential to investigate the effect of mutations in LDLR on ligand binding and/or release. Given the availability of an X-ray structure for sLDLR [22] and lipid-free apoE-NT, a detailed understanding of the molecular determinants of the binding interaction of this physiologically important ligand may be achieved in this system. Such studies should reveal the molecular properties responsible for the isoform- and lipid-dependent effects on apoE binding to LDLR family members.

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Figure 1. Characterization of recombinant apoE-NT isoforms

[A] SDS-PAGE of apoE-NT isoforms. Lane 1) molecular size standards; from top to down: 106.9, 93.6, 52.3, 37.2, 28.2 and 18.8 kDa; lane 2) apoE2-NT; 3) apoE3-NT 4) apoE4-NT. [B] Native PAGE of apoE-NT•DMPC complexes. Lane 1) the relative mobility of size standards is shown from top to bottom: Stoke's diameter = 17.0, 12.2, 9.2, 8.2 and 7.1 nm, respectively; lane 2) apoE2-NT•DMPC; lane 3) apoE3-NT•DMPC; lane 4) apoE4-NT•DMPC.

Figure 2. ApoE-NT isoform-specific binding to sLDLR

One μg AEDANS-Trp null apoE3-NT•DMPC and 2 μg sLDLR were incubated in the presence of increasing concentrations of specified unlabeled competitor apolipoprotein. Samples were excited at 280 nm and fluorescence emission intensity at 470 nm determined. [A] Lipid-free apoE2-NT (open circles) and apoE2-NT•DMPC (filled circles). [B] Lipid-free apoE3-NT (open circles) and apoE3-NT•DMPC (filled circles). [C] Lipid-free apoE4-NT (open circles) and apoE4-NT•DMPC (filled circles). [D] ApoA-I•DMPC (open squares), apoE2-NT•DMPC (filled squares), apoE3-NT•DMPC (filled triangles) and apoE4-NT•DMPC (open triangles). Values reported are the mean $\pm$ S.D. ($n = 3$).

Figure 3. Full-length apoE3 and RAP binding to sLDLR

One μg AEDANS-Trp null apoE3-NT•DMPC and 2 μg of sLDLR were incubated in the presence of increasing concentrations of a full-length apoE3 (A) or RAP (B). Samples were excited at 280 nm and fluorescence emission intensity at 470 nm determined. (A) lipid-free full-length apoE3 competitor (open circles) and full-length apoE3•DMPC competitor (filled circles). Inset (a) SDS-PAGE of recombinant full-length apoE3. The mobility of molecular size standards is shown at the left; from top to bottom: 52.3, 37.2, 28.2 and 18.8 kDa. (b) Native PAGE of full-length apoE3•DMPC. The mobility of molecular size markers of known Stoke's diameter are shown at the left; from top to bottom: 17.0, 12.2, 9.2 and 8.2 nm. (B) RAP competitor; Inset) SDS-PAGE analysis of recombinant RAP. Molecular size standards are

shown at the left from top to bottom: 94, 52 and 28 kDa. Values reported in line graphs are the mean $\pm$ S.D. (n = 3).

Figure 4. Time-dependent displacement of apoE-NT•DMPC from sLDLR

Two μg sLDLR was incubated in the presence or absence of 2 μg specified unlabeled apoE-NT•DMPC. After 0.5 min, 1 μg AEDANS-Trp null apoE3-NT•DMPC was added. Samples were excited at 280 nm and fluorescence emission intensity at 470 nm was monitored as a function of time. Trace 1) sLDLR pre-incubation in buffer only; trace 2) sLDLR plus apoE2-NT•DMPC disks; trace 3) sLDLR plus apoE4-NT•DMPC disks; trace 4) sLDLR plus apoE3-NT•DMPC disks.