Very-low-density lipoprotein binding to the apolipoprotein E receptor 2 is enhanced by lipoprotein lipase, and does not require apolipoprotein E

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The apolipoprotein (apo)E receptor 2 (apoER2) is a recently cloned member of the low-density lipoprotein (LDL) receptor (LDLR) family, showing a high homology with both the LDLR and the very-low-density lipoprotein (VLDL) receptor (VLDLR). In the present study, the binding characteristics of the apoER2 with respect to apoE and lipoprotein lipase (LPL) were investigated. VLDL was isolated from both apoE-deficient mice and mice expressing the human APOE2 (Arg¹⁵⁸ → Cys) and APOE3-Leiden isoforms on an *ApoE*^{-/-}, *Ldlr*^{-/-} double knock-out background. apoE-rich rabbit β -VLDL was used as a positive control for binding. Binding experiments performed with Chinese hamster ovary cells expressing the human apoER2 showed that the receptor was able to bind VLDL containing either of the apoE isoforms, as well as the apoE-deficient VLDL. Hence, in contrast

with the VLDLR, the apoER2 is not strictly dependent on apoE for VLDL binding. Since LPL has been shown to enhance the binding of lipoproteins to several members of the LDLR family, including the LDLR-related protein, VLDL receptor, gp330 and the LDLR itself, VLDL binding experiments were performed in the presence of LPL. Addition of LPL resulted in a significant increase in apoER2 binding for all VLDL fractions used in this study. In conclusion, lipoprotein binding of VLDL to the apoER2 is enhanced in the presence of LPL, and is not restricted to apoE-containing lipoproteins.

Key words: apolipoproteins, lipoprotein lipase, lipoprotein receptor.

INTRODUCTION

The apolipoprotein (apo)E receptor 2 (apoER2) was initially cloned from a human placenta cDNA library on the basis of homology with the coated-pit signalling domain of the low-density-lipoprotein (LDL) receptor (LDLR) family members [1]. It shows high homology with both the LDLR and the very-low-density lipoprotein (VLDL) receptor (VLDLR), even up to the level of the positioning of the exon/intron boundaries of the genes [2]. *In vitro*, the human apoER2 binds and internalizes apoE-rich rabbit β -VLDL with high affinity. The affinity for human VLDL and LDL is much lower. In humans, apoER2 expression has been detected in brain and placenta, whereas in rabbits expression has been found in brain, testis and ovary [1]. LR8b, the chicken homologue of the apoER2, is specifically expressed in brain, and binds and internalizes the activated form of α_2 -macroglobulin [3,4]. Recent findings suggest a role for the apoER2 in transmitting extracellular signals to intracellular signalling processes in a molecular pathway that regulates neuronal migration during fetal-brain development [5]. apoE has been hypothesized to play a role in modulating this signalling pathway. Since apoER2 expression levels remain high in the adult brain, it seems likely that the apoER2 is also involved in other physiologically relevant processes.

apoE plays a key role in lipid metabolism as a ligand for the receptor-mediated uptake of apoE-containing lipoproteins. It is involved in the clearance of chylomicrons, VLDL and inter-

mediate-density lipoprotein (IDL) by the liver. In addition, apoE plays a role in reverse cholesterol transport (for reviews, see [6,7]), VLDL production [8] and VLDL triacylglycerol lipolysis [9]. Despite extensive knowledge of the functions of apoE in lipoprotein metabolism, the role of apoE in lipid metabolism of the brain is poorly understood. apoE is synthesized and secreted in high levels by astrocytes, and is incorporated into phospholipid discoid particles and apoE-enriched high-density lipoprotein (HDL) residing in the cerebrospinal fluid [10]. These lipoprotein particles are thought to be taken up by brain cells via receptor-mediated endocytosis, a process in which apoERs of the LDLR family, such as the apoER2, could play a role.

Lipoprotein lipase (LPL) is the principal enzyme responsible for hydrolysis of triacylglycerols of circulating lipoproteins. Like apoE, LPL is present in the brain, and is expressed in a number of cell types, including neurons in the hippocampus, Purkinje cells of the cerebellum, and cells deep within the cortex [11]. In addition to its lipolytic activity, LPL can enhance VLDL catabolism by performing a bridging function between extracellular heparan sulphate proteoglycans and the lipoprotein particle, leading to rapid internalization via receptor-mediated pathways [12–14]. LPL can also bind directly to lipoprotein receptors of the LDLR family, and perform a bridging function between the lipoprotein particle and the receptor [15–23]. Whether LPL performs similar functions in the brain remains to be elucidated.

To study the ligand-binding characteristics of the apoER2, we

Abbreviations used: apo, apolipoprotein; apoER2, apoE receptor 2; (V)LDL(R), (very)-low-density lipoprotein (receptor); IDL, intermediate-density lipoprotein; HDL, high-density lipoprotein; LPL, lipoprotein lipase; CHO, Chinese hamster ovary.

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analysed the effect of different apoE variants on the binding of lipoproteins to the apoER2. VLDL was isolated from APOE2 mice on an *ApoE*^{-/-}, *Ldlr*^{-/-} double knock-out background. VLDL was also isolated from APOE3-Leiden mice on an *ApoE*^{-/-}, *Ldlr*^{-/-} double knock-out background. The APOE3-Leiden variant contains a tandem duplication of codons 120–126, and is associated with a dominant inheritance of familial dysbetalipoproteinaemia [24,25]. Both VLDL fractions bound to the apoER2 with high affinity. VLDL from *ApoE*^{-/-} mice also bound to the apoER2, showing that apoER2 binding is not restricted to apoE-containing lipoproteins. In addition, the effect of LPL on binding of the VLDL fractions to the apoER2 was analysed. *In vitro* binding studies revealed that LPL enhanced binding of all VLDL fractions to the apoER2 significantly.

MATERIALS AND METHODS

Mice

Ldlr^{-/-} mice were purchased from the Jackson Laboratory (Bar Harbor, ME, U.S.A.). Transgenic mice expressing human APOE2 and APOE3-Leiden in the absence of endogenous mouse apoE have been described previously [26]. *Ldlr*^{-/-}, *ApoE*^{-/-}, APOE2 and APOE3-Leiden genotypes were confirmed by PCR analysis on tail-tip DNA, as described previously [27,28]. Mice were fed *ad lib* on a regular chow diet (SRM-A; Hope Farms, Woerden, The Netherlands).

Immunoblotting

Membrane protein fractions of both apoER2-transfected and control Chinese hamster ovary (CHO) cell lines were isolated. Cells were lysed on ice by incubation in 150 mM NaCl, 10 mM EDTA, 100 mM Tris, pH 8.0, 1% (v/v) Nonidet P40 and a protease-inhibitor cocktail tablet Complete®, Mini (Boehringer Mannheim, Mannheim, Germany) in water. Cell extracts were subjected to SDS/PAGE on a 6% (w/v) polyacrylamide gel under non-reducing conditions, and transferred to nitrocellulose membranes by electrophoresis. Filters were blocked for 1 h in 3% (w/v) dried non-fat milk, 2% (v/v) normal goat serum and 0.05% (v/v) Tween 20 in PBS (blocking buffer). The membranes were then incubated in blocking buffer containing a 3000-fold dilution of the antiserum for 1 h. Subsequently, the membranes were washed three times in blocking buffer and incubated with a 1000-fold diluted goat anti-(rabbit IgG) horseradish peroxidase conjugate (Promega, Madison, NY, U.S.A.) in blocking buffer for 30 min. The membranes were then washed three times in blocking buffer, and twice in PBS. Antibody binding was detected by using the enhanced chemiluminescence detection kit (Amersham, Little Chalfont, Bucks., U.K.).

Antiserum

The antiserum used was a rabbit polyclonal antiserum generated against a synthetic peptide (EKDQFQCRN), corresponding to a segment of the first repeat of the binding domain of the apoER2, as described by Sun and Soutar [29].

Lipoprotein and LPL isolation

After a 5-h fasting period, blood was collected from *ApoE*^{-/-}, APOE2 *ApoE*^{-/-}, *Ldlr*^{-/-} and APOE3-Leiden *ApoE*^{-/-}, *Ldlr*^{-/-} mice. For the isolation of rabbit β -VLDL, a rabbit was fed on a high-cholesterol diet for at least 2 weeks, and blood was collected after a 5-h fasting period. The sera were ultracentrifuged to obtain the VLDL fractions ($d < 1.006$ g/ml). The amount of VLDL protein

was determined by the method of Lowry et al. [30]. A portion of the VLDL was labelled with ¹²⁵I with the iodine monochloride method [31]. After iodination, VLDL was dialysed extensively at 4 °C against PBS for 24 h. The apoB content of the VLDL fractions was determined by propan-2-ol precipitation, as described by Egusa et al. [32]. VLDL particle size was determined by photon correlation spectroscopy, using a Malvern 4700 C system (Malvern Instruments, Malvern, Worcs., U.K.). Measurements were performed at 25 °C, using a 90 ° angle between laser and detector.

LPL was isolated from fresh bovine milk, as described previously [33]. The isolated fraction was suspended in 20 mmol/l NaH₂PO₄/50% (v/v) glycerol, and stored in aliquots at -80 °C. Prior to the experiments, LPL was heat-inactivated at 56 °C for 30 min.

Binding studies

To study the binding of VLDL derived from transgenic mice to the apoER2, binding studies were performed using an LDL-receptor-deficient CHO cell line (IdlA7) that was stably transfected with either the human apoER2 cDNA (apoER2 CHO) or pSV2-Neo (control CHO) [1]. Binding experiments were performed essentially as described previously [34]. The cells were cultured in 24-well plates in Ham's F10 medium, supplemented with 10% (v/v) fetal-calf serum, streptomycin (200 µg/ml) and penicillin (200 units/ml) at 37 °C in air/CO₂ (19:1). Cells were washed with Ham's F10 medium three times, followed by incubation for 3 h at 4 °C with increasing amounts of ¹²⁵I-labelled VLDL in Ham's F10 medium, in either the presence or absence of a 20-fold excess of unlabelled VLDL. Specific binding was calculated as binding in the presence of excess unlabelled VLDL subtracted from binding in the absence of unlabelled VLDL. Dissociation constants were calculated by non-linear regression using GraphPad Prism (GraphPad Software Inc., San Diego, CA, U.S.A.). The binding of the different VLDL fractions in the presence or absence of LPL (5 µg/ml) was determined essentially as described previously [33]. Binding was studied at a concentration of 10 µg of total protein/ml of labelled lipoprotein, both to the apoER2 and control CHO cells. For the LPL-binding experiments, binding of VLDL to the apoER2 was defined as the amount of VLDL protein bound to the apoER2 CHO cells minus the amount of VLDL protein bound to control CHO cells.

RESULTS

Binding *in vitro* of apoE^{-/-}, apoE2 and apoE3-Leiden VLDL to the apoER2

To determine whether the human apoER2 is expressed in CHO cells, protein extracts of both apoER2 and control CHO cells were immunoblotted using rabbit polyclonal antibodies raised against the apoER2 [29]. A protein with an apparent M_r of ≈ 130 kDa was detected in the apoER2 CHO cells (Figure 1). This protein was not detected in control CHO cells, indicating that the apoER2 CHO cells do express the human apoER2.

The effect of apoE and different isoforms of apoE on lipoprotein binding to the apoER2 was studied using VLDL that was isolated from several mouse models. VLDL binding in the absence of apoE was studied using VLDL derived from *ApoE*^{-/-} mice. apoE-rich rabbit β -VLDL was used as a positive control for binding. Binding of different apoE variants to the apoER2 receptor in the absence of endogenous apoE was studied using VLDL derived from APOE3-Leiden *ApoE*^{-/-}, *Ldlr*^{-/-} and APOE2 *ApoE*^{-/-}, *Ldlr*^{-/-} mice. These mice have relatively high levels of

Figure 1 Western blot analysis of CHO cell extracts using a polyclonal antibody raised against apoER2

Membrane-protein fractions of both apoER2 CHO (apoER2) and control CHO (control) cell lines were isolated and subjected to SDS/PAGE on a 6% (w/v) polyacrylamide gel under non-reducing conditions, and subsequently transferred to a nitrocellulose membrane. The membrane was incubated with a polyclonal antiserum against the human apoER2. The position of molecular-mass markers (in kDa) run on the same gel is indicated on the left.

VLDL in their plasma, which is rich in apoE [35]. As shown in Figure 2, all ligands that were used in this study bind to the apoER2.

The amount of apoB present in the different VLDL fractions was determined by specifically precipitating apoB in these

Table 1 VLDL particle composition and its apoER2-binding characteristics

VLDL was isolated from *ApoE*^{-/-} mice (*E*^{-/-}), APO3-Leiden *ApoE*^{-/-}, *Ldlr*^{-/-} mice (*E3*-Leiden), APOE2 *ApoE*^{-/-}, *Ldlr*^{-/-} (*E2*) mice and rabbit β -VLDL. The specific binding of ¹²⁵I-labelled VLDL to the apoER2 was determined as described in the Materials and methods section. Dissociation constants were calculated by non-linear regression methods, on the basis of the amount of apoB protein, which reflects particle number. Particle size was determined by photon correlation spectroscopy. The values represent the means $\pm$ S.E.M. for three measurements. *, K_d value differs significantly ($P < 0.05$) from *E*^{-/-}, *E3*-Leiden and *E2* VLDL; †, K_d values differ significantly from rabbit β -VLDL ($P < 0.05$), as determined by Student's *t* test analysis.

VLDL isolated	Particle size (nm)	apoB (% of total protein)	K_d (μ g of apoB/ml)
β -VLDL	82 $\pm$ 18	26.3	0.67 $\pm$ 0.06*
<i>E</i> ^{-/-}	49 $\pm$ 11	34.0	10.9 $\pm$ 2.6†
<i>E3</i> -Leiden	96 $\pm$ 26	45.1	7.6 $\pm$ 0.5†
<i>E2</i>	71 $\pm$ 12	65.5	4.6 $\pm$ 0.8†

fractions. Subsequently, the protein mass of the apoB precipitate was determined (Table 1). apoB precipitation proved to be very efficient, as the percentage of radioactive label that precipitated with apoB was identical with the percentage of label present in apoB, as determined by VLDL analysis using SDS/PAGE and PhosphorImaging quantification (results not shown). K_d values, which were calculated on the basis of the amount of apoB bound to the cells, are provided in Table 1. The data show that the human apoER2 binds all VLDL fractions used in this study. The affinity of apoER2 for rabbit β -VLDL was significantly higher than that for the other VLDL fractions. No significant differences in apoER2 affinity for APOE3-Leiden, APOE2 and *ApoE*^{-/-} VLDL were found.

Figure 2 Specific binding of VLDL to apoER2-expressing and control CHO cells

Control (□) and apoER2 (●) CHO cells were incubated for 3 h at 4 °C with increasing concentrations of ¹²⁵I-labelled VLDL from *ApoE*^{-/-} mice (*E*^{-/-}), APOE3-Leiden *ApoE*^{-/-}, *Ldlr*^{-/-} mice (*E3*-Leiden), APOE2 *ApoE*^{-/-}, *Ldlr*^{-/-} (*E2*) mice and rabbit β -VLDL, in either the presence or absence of a 20-fold excess of unlabelled VLDL, as described in the Materials and methods section. Specific binding was calculated as the amount of apoB bound/mg of cell protein, which reflects particle number. Each value represents the mean $\pm$ S.D. for three experiments.

Figure 3 Effect of LPL on binding of VLDL to the apoER2

Control and apoER2 CHO cells were incubated for 3 h at 4 °C with 10 µg of total protein/ml ¹²⁵I-labelled VLDL from *ApoE*^{-/-} mice (E^{-/-}), APOE3-Leiden *ApoE*^{-/-}, *Ldlr*^{-/-} mice (E3-Leiden), APOE2 *ApoE*^{-/-}, *Ldlr*^{-/-} (E2) mice and rabbit β-VLDL. The incubations were performed in either the presence or absence of a 20-fold excess of unlabelled VLDL, with and without addition of 5 µg/ml heat-inactivated LPL. The effect of LPL on specific binding of VLDL to the apoER2 was determined by subtracting specific VLDL binding to control CHO cells from specific VLDL binding to apoER2 CHO cells. Each value represents the mean ± S.D. for three experiments. *, significantly different from binding in the absence of LPL (*P* < 0.05), as determined by Student's *t* test.

VLDL binding *in vitro* in the presence of LPL

The effect of LPL on the binding of VLDL by the apoER2 was studied with control and apoER2-expressing CHO cells. None of the VLDL fractions bound to the control cells in the absence of LPL, and also in the presence of LPL the binding capability of the control cells was low (results not shown). As shown in Figure 3, addition of LPL to apoER2 CHO cells leads to a vast increase in specific binding of all of the VLDL fractions used in the present study.

DISCUSSION

In the present study, we demonstrate that the apoER2 is, despite its name, a receptor capable of binding apoE-deficient VLDL. In addition, LPL was found to enhance VLDL binding to the apoER2. Hence the apoER2 differs from the highly homologous VLDLR, which requires apoE for lipoprotein binding. The apoER2 bound both APOE3-Leiden and APOE2 VLDL with high affinity, as was also found for the VLDLR [36,37]. High-affinity binding of these apoE variants distinguishes both the apoER2 and the VLDLR from the LDLR, which shows a severe binding defect of apoE2 and only a moderate binding defect of apoE3-Leiden [26,38]. However, neither the apoER2 nor the LDLR is dependent on apoE for lipoprotein binding.

Our experiments did not exclude a role for apoE in modulating the binding of VLDL to the apoER2. The apoER2 showed the lowest affinity for *ApoE*^{-/-} VLDL, which might indicate that apoE enhances binding. Whether differences in VLDL particle size and lipid content, as have been described previously [35] for the particles used in the present study, play an additional role in modulating binding to the apoER2 remains to be determined. LPL increased lipoprotein binding to the apoER2, as was also found for many other LDLR family members [15–19]. The LPL-mediated increase in binding was found for all VLDL fractions used in the present study.

Recently, compelling evidence has been found to support a role for both the apoER2 and the closely related VLDLR in

neuronal migration during fetal-brain development [5]. Interestingly, the VLDLR also plays a role in lipid metabolism. The VLDLR has been found to be involved in the storage of lipids in fat tissue [39]. In addition, the VLDLR has been found to lower serum cholesterol levels when ectopically expressed in the liver of several hypercholesterolaemic mouse models, indicating that the VLDLR is capable of lipid transport from the circulation into the cells [36,40]. In the present paper, we show that the apoER2 displays distinct, but overlapping, lipoprotein-binding characteristics as compared with the VLDLR, suggesting that the apoER2 has a similar dual function in neuronal migration and lipid metabolism.

Both the apoER2 and LPL have been shown to be expressed in the cerebral cortex, cerebellum and hippocampus [1,11,41]. The chicken homologue of the apoER2 is expressed in neurons throughout the brain, including the Purkinje cells of the cerebellum, and in cells that constitute brain barriers, such as endothelial cells of blood vessels, cells of the arachnoid membrane, and cells of the choroid plexus [4]. LPL in the brain is also expressed by neurons, including the Purkinje cells, and LPL protein is found in many vascular structures throughout the central nervous system [11]. The similarities in expression pattern between LPL and the apoER2, together with the data presented in the present paper on enhanced lipoprotein binding to the apoER2 by LPL, support a role for the apoER2 in lipid metabolism of the brain. On the basis of its localization, it may be hypothesized that the apoER2 is involved in transport of lipids into neurons and across brain barriers, a process that could be facilitated by LPL.

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