reported that LDL receptor with the deletion of the exon encoding the sixth repeat, which has no recognition by IgG-C7, abolishes the binding of LDL but not βVLDL (Hobbs et al. 1986; Russell et al. 1989b). They concluded that the fifth cysteine-rich repeat of the ligand-binding domain would be a crucial role for binding of LDL but not βVLDL. Our results showed that the 25S-LDLR bound LDL as well as βVLDL with high affinity and took up βVLDL more rapidly than LDL. These results indicate that the disulfide bond of the fourth cysteine of the first ligand-binding domain might also be a crucial role for binding and uptake of atherogenic lipoproteins, such as remnant lipoproteins and chylomicron remnants, despite reduced LDL uptake, and suggest the enhancement of progression for CHD.

Autosomal-recessive hypercholesterolemia, which is caused by a mutation of a putative LDL receptor adaptor protein, has been recently identified. Studies of this disorder have shown that signaling through the adaptor protein in the cytosol is required for the endocytosis of receptor-bound LDL (Garcia et al. 2001). Decreased uptake of LDL or βVLDL via the mutant receptor may be affected by defect signaling for the endocytosis. Although the mutant 25S-LDLR had the similar binding activity for LDL as the wild-type LDLR and mutant K790X LDLR, which is lacking the cytoplasmic domain involving the phosphotyrosine binding (PTB) domain (Garcia et al. 2001) and has defective endocytosis of LDL, the 25S-mutant had defective uptake of LDL like the 790X-mutant. These results suggest that the NH_2 terminus of the ligand-binding domain may have a role in signaling for the endocytosis. The precise mechanism needs further investigation.

In addition, the LDLR binds apoB-100 and apoE, whereas the VLDLR or apoER2 binds only apoE (Takahashi et al. 1992, 1996; Kim et al. 1996). The phenotype of apoE has no effect on binding to the VLDLR (Bieri et al. 1998), and the ligand-binding site of apoE has not been identified. These considerations suggest that LDLR binds LDL and βVLDL at different sites. Our results showed that uptake of LDL but not βVLDL was affected in the mutant receptor, suggesting that the cytoplasmic signaling for the endocytosis may be mediated by at least two or more systems. The precise mechanism also needs further investigation.

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Sources of Acetyl-CoA: Acetyl-CoA Synthetase1 and 2

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Abstract: Acetyl-CoA synthetase (AceCS) catalyzes the production of acetyl-CoA from acetate, CoA and ATP. There are two types of AceCS in mammals with different functions. One designated AceCS1 is a cytosolic enzyme expressed in the liver and plays a role in the production of acetyl-CoA for the synthesis of fatty acids and cholesterol. The other enzyme AceCS2 is a mitochondrial matrix enzyme that produces acetyl-CoAs mainly utilized for oxidation. Consistent with its function, the transcription of AceCS1 is regulated by SREBPs. In contrast, the expression of AceCS2 is upregulated during starvation and ketogenesis via unknown mechanisms. Specific inhibitors of AceCS may provide therapeutic agents for the treatment of obesity, cardiovascular diseases and type 2 diabetes.

INTORODUCTION

Acetyl-CoA synthetase (AceCS, EC6.2.1.1) catalyzes the ligation of acetate and CoA to produce acetyl-CoA, an essential molecule utilized in various metabolic pathways including fatty acid and cholesterol synthesis and the TCA cycle (reviewed in Ref. [1]).

Although AceCS has been studied extensively in *Neurospora*, aspergillus and *S. cerevisiae*, the mammalian enzyme has been poorly characterized until recently. In contrast to animal cells, yeast can grow on acetate as a sole carbon source because they can convert acetate to glucose through the glyoxylate cycle, which requires that acetate first be activated to the CoA derivative [2]. Yeast cells have two distinct AceCS genes encoding 57% identical proteins [3]. Disruption of both AceCS genes in yeast is lethal, even when the cells are grown on glucose. This lethality is attributed to the fact that yeast derives most of its endogenous acetyl-CoA from decarboxylation of pyruvate, which produces acetate that must be activated to acetyl-CoA for synthesis of fatty acids [3].

In contrast to yeast, animal cells produce acetyl-CoA from two mechanisms that do not require AceCS: i) pyruvate dehydrogenase, which converts pyruvate to acetyl-CoA without generating free acetate, and ii) β-oxidation of fatty acids, which also produces acetyl-CoA as an end product. These reactions occur predominantly in mitochondria. Under aerobic conditions, most of the acetyl-CoA is oxidized within mitochondria through the TCA to produce energy. For fatty acid biosynthesis, acetyl-CoA produced in the mitochondria must be transported to the cytoplasm. This transport is effected by the condensation of acetyl-CoA with oxaloacetate within mitochondria to form citrate, which is then transported to the cytoplasm where it is converted to acetyl-CoA by ATP citrate lyase.

Although acetate is not an essential source of acetyl-CoA in animals, AceCS plays a key role in the catabolism of acetate generated by several conditions. i) Acetate is generated in the colon by bacterial fermentation, and this production is increased by a high fiber diet [4, 5]. The acetate is transported via the portal vein to the liver. ii) ingested ethanol is oxidized in liver, first to acetaldehyde and then to acetate. iii) An appreciable amount of acetate is generated from acetyl-CoA in the cytoplasm by acetyl-CoA hydrolase [6]. iv) Acetate is generated within the nucleus of all cells by histone deacetylases [7]. v) In the nervous system, AceCS is postulated to play a key role in the recycling of acetate released by acetylcholine esterase for the formation and release of acetylcholine in the cholinergic nerve terminals [8, 9].

AceCS from various microorganisms are shown to belong to the firefly luciferase super family [10, 11] that includes mammalian long-chain acyl-CoA synthetases, ACS1-ACS5 [12-16], bacterial antibiotic synthetases, 4-coumarate: CoA ligases, and luciferases from various origin. All enzymes in this enzyme family contain a common sequence motif of Ser-Gly-(small hydrophilic residue)2-Gly-(any residue)-Pro-Lys-Gly and catalyze two step common reactions: adenylation of substrates and the subsequent thioester formation [10, 11].

To evaluate the role of AceCS in mammals, we isolated and characterized two separate AceCSs of human and murine origin [18, 28]. In this review, we describe molecular and enzymatic properties, and regulation of the enzymes, and discuss their roles in animals.

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TWO ACECSS IN MAMMAL

A Mammalian AceCS cDNA was first identified by Luong *et al.* [17], using a suppression subtractive hybridization method from Chinese hamster ovary cells that expressed high levels of nuclear forms of sterol regulatory element-binding proteins (SREBPs). Independently, we isolated two cDNAs that encode functionally distinct murine acetyl-CoA synthetases. One cDNA, designated AceCS1, encodes a cytosolic enzyme identical to the cDNA isolated by Luong *et al.* [17]. The other cDNA, AceCS 2, encodes a mitochondrial matrix enzyme that contains a putative mitochondrial-targeting signal [18]. The predicted amino acid identity between AceCS1 and AceCS2 is 45.8%, and both enzymes exhibit similar affinity for acetate.

The mRNA for AceCS1 is expressed as a single species of mRNA (~3 kilobases in length) that is most abundant in the liver and kidney. Lower levels were seen in the heart, and testis. The mRNA is not found in spleen lung, and skeletal muscle. In contrast, The AceCS2 transcripts are expressed in a wide range of tissues, with the highest level in heart, relatively high levels in spleen, lung, skeletal muscle, kidney and testis, and lower levels in the brain.

The subcellular fractionation of a mouse kidney homogenate revealed that AceCS1 is detected exclusively in the cytosolic fraction, whereas AceCS2 is present mainly in the light and heavy mitochondrial fractions. Together with the solubility in hypotonic buffer of AceCS2 and the presence of CoA pool in the mitochondrial matrix, our cell fractionation study indicated that AceCS2 is a mitochondrial matrix enzyme.

REGULATION OF ACECS1 BY SREBPS

AceCS1 is a member of a family of genes whose transcription is regulated by SREBPs, the basic helix-loop-helix leucine zipper family of transcription factors. SREBPs activate multiple genes that are required for cholesterol and unsaturated fatty acid metabolism: 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-CoA synthase), HMG-CoA reductase, farnesyl diphosphate synthase, squalene synthase, lanosterol demethylase, and others [19, 20]. SREBPs are synthesized as membrane-bound precursors with two transmembrane domains. Upon sterol deprivation, SREBPs are activated to release the transcriptionally active NH_2-terminal segment through a mechanism requiring two sequential proteolytic events [21]. This proteolytic cleavage is regulated by SREBP cleavage-activating protein (SCAP), which forms complexes with SREBPs and guides them from the endoplasmic reticulum to the Golgi complex [22-25], where each SREBP is cleaved sequentially by Site-1 protease (S1P) [26] followed by Site-2 protease (S2P) [27]. The mature SREBPs corresponding to the NH2-terminal half of the complete proteins are translocated to the nucleus, where they bind to sterol regulatory elements (SREs) and, in cooperation with generic transcription factors such as Sp1 and nuclear factor-Y (NF-Y), they activate transcription of genes involved in cholesterol and fatty acid synthesis.

Like the pattern of regulation of genes involved in fatty acid synthesis, AceCS1 mRNA was induced when cultured cells were deprived of sterols and negatively regulated by sterol addition. Similarly, marked induction of AceCS1 mRNA and protein were seen during the differentiation of 3T3-L1 cells. AceCS1 mRNA was also elevated in livers of transgenic mice that express dominant-positive versions of all three isoforms of SREBP, SREBP-1a, -1c and -2.

The promoter region of the murine AceCS1 gene has a unique structure consisting of an E-box, two putative CAAT-boxes, eight copies of the SRE, and six GC-boxes. All eight SREs bind purified SREBP-1a [28], and all eight are required for a maximal response to co-transfected SREBP. Additionally, three of them are absolutely required for the normal activation that occurs when cultured cells are deprived of sterols [28]. Seven of these SRE sites resemble the SRE-1 present in the promoter for the LDL receptor gene, and one of them more closely resembles the SRE present in the rat FAS promoter and the SRE-3 element present in the promoter of the FPP gene. A number of genes have been shown to be regulated at the transcriptional level by mature SREBPs. However, the AceCS1gene represents the first case in which a tandem array of up to eight SREBP sites are required for maximal activation by SREBP.

To achieve high levels of gene activation, the regulation by SREBPs require additional co-regulatory transcription factors, including Sp1, Sp2 and NF-Y. In the LDL receptor and FAS promoters, an Sp1 binding site located adjacent to the SRE elements is crucial for SREBP-mediated promoter activation, and nuclear accumulation of SREBPs results in an increased association of Sp1 and enhances the binding of Sp1 [29,30] to its site. In the AceCS1 promoter, there are six copies of potential Sp1 binding sites (GC-boxes) 3' to the SRE cluster. Among these GC-boxes, the most 5' GC-box is adjacent to the most 3' SRE and this GC box is critically important in sterol-regulated transcription. This critical GC-box actually binds both Sp1 and Sp3 as revealed by a gel supershift analysis with nuclear extracts from HepG2 cells. In addition, transfection assays in *Drosophila* SL2 cells, which is an ideal cell-based assay system to examine the requirement for Sp1 or NF-Y [31, 32], demonstrated that SREBP synergistically stimulated the *AceCS1* promoter together with Sp1 or Sp3 but not with nuclear factor-Y [28].

REGULATION OF ACECS2 UNDER KETOGENIC CONDITIONS

AceCS2 is highly expressed in the heart and skeletal muscle and the levels of the mRNAs in the heart and skeletal muscle are regulated under ketogenic conditions [18]. A marked induction of AceCS2 mRNA was seen in the heart, and skeletal muscle when animals are starved [18]. In contrast, the AceCS1 mRNA level in the skeletal muscle decreased by approximately 50%, whereas no changes were seen in the heart. The levels of AceCS2 mRNA in the skeletal muscle of Zucker diabetic fatty rats were approximately 3-fold higher than those in the normal littermates, whereas almost no changes were found in the levels of AceCS1 mRNA. These data indicated that the AceCS2 transcripts are induced in the heart and skeletal muscle under ketogenic conditions.

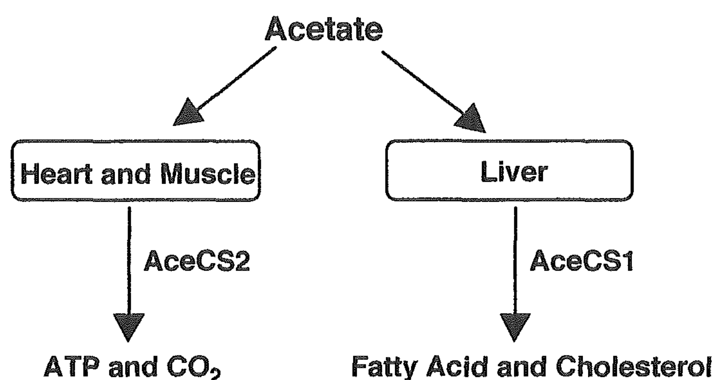

Fig. (1). A model representing differential roles of two different AceCSs in acetate metabolism. AceCS1 is a cytosolic enzyme that is upregulated by SREBPs and provides acetyl-CoA for the synthesis of fatty acids and cholesterol. In contrast, AceCS2 is a mitochondrial matrix enzyme that is induced under ketogenic conditions, generates acetyl-CoA for energy production.

Under ketogenic conditions, relatively large amounts of free acetate are released from the liver from fatty acids [33]. Consistent with the increased production of acetate, plasma levels of acetate are also increased by starvation and in patients with diabetes [4, 34]. Furthermore, the hepatic activities of cytosolic acetyl-CoA dehydrogenease is also induced under ketogenic conditions [35]. The acetate release from the liver under ketogenic conditions must be ligated to CoASH by AceCS in order for it to be further metabolized in extrahepatic tissues. AceCS2 is highly expressed and induced by ketogenic conditions in both heart and skeletal muscle. It is also not expressed at any appreciable levels in the liver. These observations strongly suggest that AceCS2 plays a key role in the metabolism of acetate for energy production under ketogenic conditions predominantly in skeletal muscle and heart. Consistent with our hypothesis, AceCS2 is an abundant protein in the heart of ruminant mammals [36, 37], where large amounts of acetate are produced by microorganisms that are present in plant fibers in the rumen [1]. The molecular mechanism underlining the induction of AceCS2 mRNA under ketogenic conditions remain unclarified.

ALLICIN, A SPECIFIC INHIBITOR OF ACECS

There are several known AceCS inhibitors including allicin, adenosine 5'-alkylphospates (analogs of acyl adenylates), and orotic acid. Among these componds, allicin is a specific inhibitor for AceCS [38]. Allicin is a naturally occurring antibiotic from garlic and specifically inhibits AceCS activity with an I_{50}-value lower than 10 μM [38]. Allicin and the development of its derivatives may provide novel therapeutic agents for the treatment of obesity, cardiovascular diseases and type 2 diabetes.

METABOLIC FATE OF ACETATE ACTIVATED BY TWO ACECSS

Using AceCS1 overproducing cells, Luong *et al.* demonstrated that AceCS1 provides acetyl-CoA for the synthesis of fatty acids and cholesterol [17]. Similarly, we incubated AceCS2 transfected cells with labeled-acetate and showed that the major function of AceCS2 is to produce acetyl-CoA for oxidation through the TCA cycle to produce ATP and CO_2 in the mitochondrial matrix [18]. These results demonstrate that there are two acetyl-CoA pools derived from acetate: one is a cytoplasmic pool, generated by AceCS1, which is exclusively utilized for the synthesis of fatty acids and cholesterol: and the other is a mitochondrial matrix pool, generated by AceCS2, that is mainly oxidized for energy production by oxidation (Fig. **1**). Now that the genes encoding two AceCSs have been identified, it will be of interest to explore the physiologic roles of the enzymes in liver and brain in response to ethanol and other sources of acetate. Further studies are necessary to elucidate precise function and regulation of the two enzymes, and to determine disorders caused by the absence of the two enzymes.

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The very low density lipoprotein (VLDL) receptor – a peripheral lipoprotein receptor for remnant lipoproteins into fatty acid active tissues

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Abstract

The VLDL (very low density lipoprotein) receptor is a member of the LDL (low density lipoprotein) receptor family. The VLDL receptor binds apolipoprotein (apo) E but not apo B, and is expressed in fatty acid active tissues (heart, muscle, adipose) and macrophages abundantly. Lipoprotein lipase (LPL) modulates the binding of triglyceride (TG)-rich lipoprotein particles to the VLDL receptor. By the unique ligand specificity, VLDL receptor practically appeared to function as IDL (intermediate density lipoprotein) and chylomicron remnant receptor in peripheral tissues in concert with LPL. In contrast to LDL receptor, the VLDL receptor expression is not down regulated by lipoproteins. Recently several possible functions of the VLDL receptor have been reported in lipoprotein metabolism, atherosclerosis, obesity/insulin resistance, cardiac fatty acid metabolism and neuronal migration. The gene therapy of VLDL receptor into the LDL receptor knockout mice liver showed a benefit effect for lipoprotein metabolism and atherosclerosis. Further researches about the VLDL receptor function will be needed in the future. (Mol Cell Biochem **248**: 121–127, 2003)

Key words: VLDL receptor, atherosclerosis, obesity/insulin resistance, fatty acid metabolism, gene therapy, neuronal migration

Introduction

LDL receptor family is growing more than 10 receptors. In addition to the LDL receptor itself [1], LDL receptor-related protein (LRP) [2], megalin (GP330) [3], the VLDL receptor [4], apoE receptor 2 (apoER2) [5], sorLA-1/LR11 [6], LRP3 [7], LRP4 [8], LRP5 [9], LRP6 [10], LRP1B/LRP-DIT (deleted in tumors) [11], and LRP9 [12] cDNA have been cloned. Among these receptors, the VLDL receptor and apoER2 are most structurally similar to the LDL receptor. In this review, we will focus on studies about the VLDL receptor mainly and discuss recently elucidated physiological functions of the VLDL receptor.

LDL receptor

Lipoprotein receptors play a central role of cholesterol homeostasis in the body. First low-density lipoprotein (LDL) receptor, whose genetic defect induces familial hypercholesterolemia (FH) has been discovered and elucidated as a LDL receptor pathway [13]. Plasma LDL particles are recognized, internalized and degraded by the coated-pit located hepatic LDL receptors. FH heterozygous (about 1 per 500 people) express half of the normal number of functional LDL receptor and rare FH homozygous (about 1 per million people) express few to no functional LDL receptors on their cell surface. Their plasma cholesterol level rise 300–500 and 600–

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1200 mg/dl respectively. FH homozygous frequently die of heart attack before 20 years of age because excess lipids are accumulated in their coronary arterial walls. The human LDL receptor mRNA, 5.3-kilobases (kb) in length, encodes a protein of 860 amino acids [1]. The human LDL receptor gene, located on chromosome 19, spans 45-kb and is divided into 18 exons and 17 introns. LDL receptor protein is composed of five domains: (i) an amino-terminal, ligand-binding domain composed of multiple cysteine-rich repeats; (ii) an epidermal growth factor (EGF) precursor homology domain; (iii) an O-linked sugar domain with clustered serine and threonine residues; (iv) a transmembrane domain; and (v) a cytoplasmic domain with an NPVY sequence (Fig. 1). More than 150 different mutant alleles have been detected among FH patients. LDL receptor binds two proteins: (i) apoB-100, the 400,000-dalton glycoprotein that is the sole protein of LDL; and (ii) apo E, a 34,000-dalton protein that is found in multiple copies in VLDL and IDL. Ligand binding specificity indicates that LDL receptor binds VLDL, IDL and LDL but not HDL (high density lipoprotein) and oxidized LDL as lipoprotein particles. When cells catch up LDL particles into cytoplasm through LDL receptor pathway, three steps are going to act to stabilize the cell's cholesterol content: (i) a suppression of HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase gene (a key enzyme for de novo cholesterol synthesis) and acceleration of the degradation of the enzyme protein (ii) an activation of ACAT (Acyl-CoA: cholesterol acyltransferase, a cholesterol-esterifying enzyme) to protect cells from free cholesterol (iii) a suppression of LDL receptor own gene (a key lipoprotein receptor taking up from plasma cholesterol). It has been accepted that LDL receptor expression is down regulated by intracellular cholesterol content to prevent from entering excess cholesterol into cells.

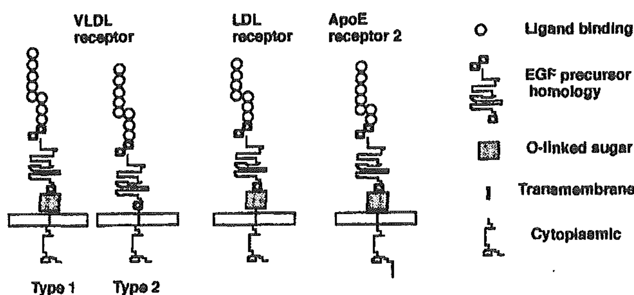

Fig. 1. Schematic model of VLDL receptor, LDL receptor and apo E receptor 2. Note that the VLDL receptor and apoER2 are similar to the LDL receptor structurally and three lipoprotein receptors are composed of five domains.

Discovery of the VLDL receptor

In 1992, we cloned and characterized a new lipoprotein receptor designated VLDL receptor from a rabbit heart cDNA library [4]. We speculated that probably another lipoprotein receptor more than LDL receptor would have a similar sequence and structure. To exclude the rabbit LDL receptor, the entire pooled cDNA library was digested with Sal I and recircularized with T4 DNA ligase. The presence of a unique Sal I site in the rabbit LDL receptor cDNA and the Okayama-Berg vector resulted in loss of any LDL receptor cDNAs after recircularization and retransformation. The resulting LDL receptor-subtracted cDNA library was screened with the 1.9-kb Sma I-Sal I fragment from the rabbit LDL receptor cDNA under low-stringency hybridization conditions. Surprisingly a cloned new cDNA encodes a protein with striking homology to the LDL receptor (Fig. 1). The mature protein consists of the following five domains spanning 846 amino acids: 328 N-terminal amino acids including an 8-fold repeat of 40 amino acids homologous to the ligand binding repeat of the LDL receptor; 396 amino acid residues homologous to the epidermal growth factor precursor including three cysteinrich repeats; a region immediately outside of the plasma membrane rich in serins and threonines; 22 amino acids transversing the plasma membrane; and 54 amino acids including the NPVY sequence that is required for clustering of the LDL receptor in coated pits and that projects into the cytoplasm. Following the rabbit VLDL receptor cDNA cloning, we also cloned the human VLDL receptor cDNA from THP-1 monocytic leukemia cells cDNA library [15]. The human VLDL receptor gene contains 19 exons spanning approximately 40-kb. The exon-intron organization of the gene is almost the same as that of the LDL receptor gene, except for an extra exon that encodes an additional repeat in the ligand-binding domain (LDL receptor contains a 7-fold repeats and VLDL receptor has an 8-fold repeats). The VLDL receptor mRNAs produce two kinds of VLDL receptor proteins by alternative splicing, type 1 VLDL receptor and type 2 VLDL receptor that lacks O-linked sugar domain encoded by exon 16. Although the structure and organization of the VLDL receptor gene is highly similar to the LDL receptor gene, the two genes are located on different chromosomes: the LDL receptor gene on chromosome 19 and the VLDL receptor gene on chromosome 9. The VLDL receptor mRNAs are highly abundant in heart, muscle and adipose tissue and barely detectable in liver that the LDL receptors are expressed abundantly. We suggested that the VLDL receptor might mediate TG-rich lipoproteins into peripheral fatty acid active tissues [14].

Unique ligand binding specificity of the VLDL receptor

To confirm the ligand binding specificity of the new cloned gene that was similar to the LDL receptor structurally, we transfected the cloned cDNA into ldlA-7 cells (LDL receptor deficient CHO cells) and examined the ligand binding specificity compared to LDL receptor transfectants. The new gene producing proteins bound apoE-containing lipoproteins including VLDL, IDL from WHHL (Watanabe heritable hyperlipidemic) rabbits and β–VLDL (β-migrating VLDL) from cholesterol-fed rabbits, but did not bind LDL from WHHL rabbits, whereas CHO cells transfected with the human LDL receptor cDNA bound both apoB- and apoE-containing lipoprotein including VLDL, IDL, LDL from WHHL rabbits and β–VLDL from cholesterol-fed rabbits. ^{125}I-labeled β–VLDL binding to the transfected cells was inhibited by unlabeled apo E-liposomes indicating that the receptor recognizes apo E [4, 14, 15]. On the other hand, VLDL from fasted normal human subjects bound with lower affinity than VLDL prepared from WHHL rabbits or β–VLDL from cholesterol-fed rabbits. The low affinity binding of fasted human VLDL to the VLDL receptor can be overcome by enriching VLDL with either apo E or LPL [16]. There are three mechanisms between LPL and the VLDL receptor: (i) directly binding to the receptor that was confirmed by Argraves KM *et al.* [17] (ii) mediating the binding lipoprotein particles to heparan sulfate proteoglycans prior to interaction with the receptor (iii) its lipolytic activity, converting VLDL particles to smaller remnants (apo E-rich particles) before these can become endocytosed by receptors. Following our findings of unique ligand binding specificity of the VLDL receptor for VLDL particles, Beisiegel *et al.* [18] showed the same mechanism was the case for chylomicron particles. Taking into account that the VLDL receptor and LPL are expressed in the same tissues, these findings suggest that the metabolism of IDL (VLDL remnant) and chylomicron remnant (CR) could be mediated by the VLDL receptor in peripheral fatty acid active tissues in concert with LPL (Fig. 2).

In contrast to the ligand binding specificity of the VLDL receptor *in vitro*, the VLDL receptor knockout (VLDL-R–/–) mice did not show any lipoprotein abnormality although adipose tissue mass of VLDL-R–/– mice was reduced [19]. Tacken *et al.* [20] demonstrated that the VLDL receptor is a peripheral lipoprotein receptor for VLDL triglycerides *in vivo*. They created the VLDL receptor/LDL receptor double knockout mice (VLDL-R–/–; LDL-R–/–) mice and VLDL receptor-overexpressing mice on LDL receptor knockout (LDL-R–/–) mice. When mice were fed a high fat diet, absence of the VLDL receptor (VLDL-R–/–; LDL-R–/–) resulted in a significant increase in serum triglyceride level, and

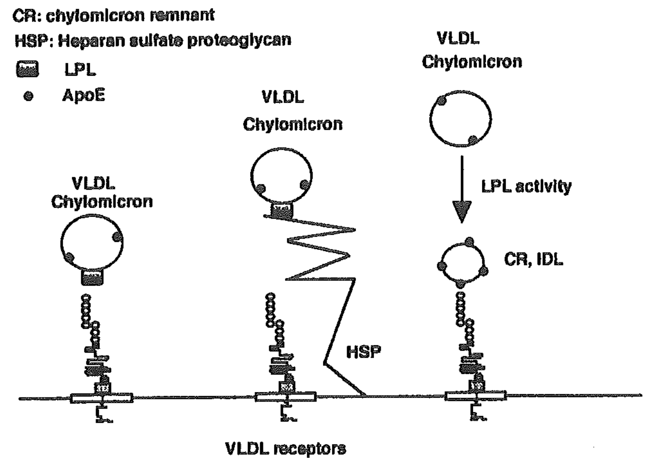

Fig. 2. Possible mechanism of LPL on the binding of TG-rich lipoproteins to the VLDL receptor. VLDL receptor is involved in the metabolism of remnant lipoprotein particles into peripheral fatty acid active tissues in cooperation with LPL.

overexpression of the VLDL-R resulted in a significant decrease in serum triglyceride levels compared to LDL-R–/– mice. Furthermore, a period of prolonged fasting in a chow diet showed a significant increase in serum triglyceride levels in VLDL-R–/–; LDL-R–/– mice compared to LDL-R –/– mice. Our hypothesis that the VLDL receptor plays a role in lipid metabolism for triglycerides dates from almost a decade ago has been proven *in vivo*.

Atherosclerosis and VLDL receptor

Familial hypercholesterolemia (FH) patients lacking LDL receptor accumulate massive lipids in macrophages indicating that LDL receptor is not involved in macrophage foam cell formation. It has been proposed that there are two receptors that contribute to macrophage foam cell formation, scavenger receptors and β–VLDL receptor. We speculated that the VLDL receptor might be a so-called macrophage β–VLDL receptor by its ligand binding specificity. We reported that the VLDL receptor on THP-1 cells and rabbit alveolar macrophages was not down-regulated by incubation with β-VLDL [15, 21]. Moreover, incubation of β-VLDL with LDL-deficient CHO cells (ldlA-7) transfected with the rabbit VLDL receptor enabled these cells to accumulate cholesteryl ester, resulting in foam cell formation [21]. Both apoE2/2 VLDL and apoE3/3 VLDL were recognized by the VLDL receptor identically [22]. 1α, 25-dihydroxyvitamin D_3 induces VLDL receptor mRNA expression in HL-60 cells in association with monocytic differentiation [23]. Strickland DK and colleagues reported that the atherogenic lipoprotein Lp (a) was also rec-

ognized by the human VLDL receptor [24]. VLDL receptor expression, primarily in macrophages, has been confirmed in human and rabbit atherosclerotic lesions [24–27]. Those finding encouraged us to imagine that the VLDL receptor might be a crucial role for macrophage foam cell formation especially in diabetes mellitus and type III hyperlipoproteinemia. We looked for some agents that inhibited the expression of the VLDL receptor. We found that IFN (interferon)-γ inhibited VLDL receptor expression in a dose-and time-dependent manner in macrophages. In THP-1 macrophages, VLDL receptor protein expression decreased at 2 days after PMA-treatment, but increased at 3 days and increased up to 5 days. ^{125}I-β-VLDL degradation study and Oil red-O staining showed that IFN-γ significantly inhibited foam cell formation following uptake of β-VLDL (remnant lipoprotein). LRP and LDL receptor expression that bound β-VLDL were not expressed in THP-1 macrophages. In PMA-treated HL-60 macrophages and human monocyte-derived macrophages, IFN-γ also inhibited VLDL receptor expression and foam cell formation by β-VLDL [28]. Those data showed that the VLDL receptor might be a macrophage β-VLDL receptor even though we used human macrophage cells *in vitro*. But the controversial finding using mouse model *in vivo* was reported. Yagyu *et al.* [29] showed that atherosclerosis was not different between HuB (human apo B) transgenic mice and VLDL receptor deficient HuB transgenic mice after 4 months of an atherogenic diet. They also detected that VLDL receptor deficient induced low LPL activity. As mentioned above, LPL activity is an important factor for the binding of TG-rich lipoproteins to the VLDL receptor. One explanation for the difference is that low LPL activity may reduce the uptake of TG-rich lipoproteins into macrophages in VLDL receptor *deficient HuB transgenic mice. Second explanation is that* there are some different factors between human and mouse atherosclerotic lesions. In fact, we are not able to detect an enough amount of VLDL receptor expression in mouse peritoneal macrophages and J774 mouse macrophage cells. But further experiments are needed to dissolve this problem.

Obesity and VLDL receptor

VLDL receptor knockout (VLDL-R–/–) mice showed a modest decrease in adipose tissue mass [19]. Recently Goudriaan *et al.* [30] reported that VLDL-R–/– mice remained lean and prevented from insulin resistance after 17 weeks of high-fat, high-calorie diet compared to wild-type mice, and the weight gain of VLDL-R–/– on ob/ob mice was less profound compared with ob/ob mice. Those data indicate that inhibition of VLDL receptor expression in adipose tissue may be a therapeutic strategy for multiple risk factor syndrome.

Gene therapy for familial hypercholesterolemia (FH) using VLDL receptor

Chan *et al.* [31] reported that adenovirus-mediated transfer of the VLDL receptor gene into LDL receptor knockout mice liver induced greatly enhanced the ability to clear IDL fraction, resulting in a marked lowering of the plasma IDL/LDL fraction. Moreover they also showed that helper-dependent adenovirus-mediated delivery of VLDL receptor into hepatocytes produced long-term lowering of plasma cholesterol and prevented atherosclerosis development in LDL receptor knockout mice in 2001 [32]. The gene therapy using VLDL receptor instead of LDL receptor for FH is reasonable because the normal LDL receptor might be recognized as a foreign protein in FH patients. The VLDL receptor is non-immunogenic because VLDL receptor is normally expressed in fatty acid active tissues even in FH patients. We are looking forward to hearing the great boon for FH homozygous using VLDL receptor gene therapy in the near future.

VLDL receptor and cardiac fatty acid metabolism

The most abundant tissue of the VLDL receptor expression is the heart. Long-chain fatty acids are important substrates for energy production in both the heart and skeletal muscle. In the adult heart, fatty acids are the preferred energy substrates. The rabbit heart switches from glucose to fatty acids as an energy substrate within the first week after birth [33]. Two mechanisms contribute to delivery of substrate (fatty acid transport) into the heart [34]. Simple diffusion functions significantly at high molecular ratios of fatty acid to albumin when the concentration of fatty acid in solution is increased. The second mechanism depends on FAT (fatty acid translocase)/CD36, FATP (fatty acid transport protein) and FABPpm (plasmalemmal fatty acid-binding protein), which have been established as membrane-bound fatty acid transporters to the heart. Indirect evidence showing that the VLDL receptor may play a pivotal role in cardiac metabolism has been reported. Kraemer *et al.* [35] showed that the VLDL receptor protein expression in Balb/c mice heart increased progressively with fasting and proposed a potential role for the VLDL receptor in the delivery of triglycerides/fatty acids as fuel. Using SHRs-SP (spontaneously hypertensive rats-stroke prone), Masuzaki *et al.* [36] reported that ventricular VLDL receptor mRNA decreased when cardiac hypertrophy was established. The reduced VLDL receptor expression in SHRs-SP after cardiac hypertrophy might be linked with the switch in energy

substrate from lipid to glucose known to occur in cardiac hypertrophy. But the direct evidence showing lipoprotein uptake into myocardium has not been reported. We attempted to elucidate the relation between the VLDL receptor and cardiac fatty acid metabolism [37]. In the Balb/c fasting mice for 48 h, VLDL receptor as well as LPL, FAT/CD36, H-FABP (heart-type fatty acid-binding protein), ACS (acyl-CoA synthetase) and LCAD (long chain acyl-CoA dehydrogenase) mRNAs for fatty acid metabolism indicators increased. PK (pyruvate kinase) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNAs for glucose metabolism indicators did not changed. Electron microscopic examination indicated the lipid droplets accumulated in the hearts of fasting Balb/c mice for 48 h. During the development of SD (Sprague-Dawley) rats, VLDL receptor, LPL, FAT/CD36, H-FABP, ACS and LCAD mRNAs increased gradually with growth. However, PK and GAPDH mRNAs did not show this tendency. In cultured neonatal rat cardiomyocytes, VLDL receptor expression increased with days in culture, which was compatible to *in vivo* results. Oil red-O staining showed cardiomyocytes after 7 days in culture (when the VLDL receptor protein is present) accumulated β-VLDL. There were no detectable LDL receptor mRNAs in cultured neonatal rat cardiomyocytes showing that remnant lipoproteins (β-VLDL) are taken up to myocardium through the VLDL receptor. Triglycerides hydrolysis by LPL results in the generation of free fatty acids, 2-monoglycerol and remnant lipoprotein particles (IDL and chylomicron remnant). Resultant fatty acids are transported across plasma membrane into heart by simple diffusion and membrane-associated transporters (FAT/CD36, FATP and FABPpm). On the other hand, remnant lipoprotein particles are also taken up into heart by VLDL receptor (Fig. 3). Detailed studies of the VLDL receptor in relation to fatty acid metabolism and in response to various nutritional conditions should be examined.

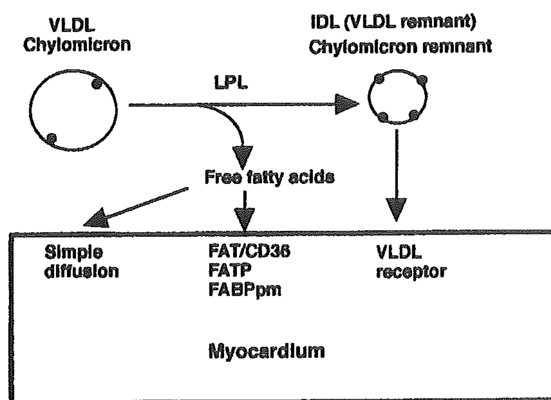

Fig. 3. Novel pathway for cardiac fatty acid metabolism by the VLDL receptor. LPL produces free fatty acids, glycerol and remnant lipoprotein particles. Free fatty acids are taken up into myocardium by simple diffusion and fatty acid transporters. On the other hand, remnant lipoprotein particles are also taken up through the VLDL receptor pathway.

Other ligands of the VLDL receptor

In addition to apo E and LPL, the VLDL receptor binds RAP (receptor-associated protein) [38], thrombospondin-1 [39], urokinase plasminogen activator (uPA)/plasminogen activator inhibitor-1 complex [17, 40], several other proteinase-serpin complexes [41], and Reelin [42–44]. It was surprised that VLDL receptor and apoER2 are involved in the Reelin signaling pathway and neuronal migration. Both VLDL receptor and apoER2 can bind Reelin on their extracellular domains, which subsequently induces tyrosine phosphorylation of mDab-1 (mammalian Disabled-1). Ten years have passed since the discovery of the VLDL receptor. Research of the VLDL receptor will continue to expand in the field of the brain.

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Severe Hypercholesterolemia, Impaired Fat Tolerance, and Advanced Atherosclerosis in Mice Lacking Both Low Density Lipoprotein Receptor-related Protein 5 and Apolipoprotein E*

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LDL receptor-related protein 5 (LRP5) plays multiple roles, including embryonic development and bone accrual development. Recently, we demonstrated that LRP5 is also required for normal cholesterol metabolism and glucose-induced insulin secretion. To further define the role of LRP5 in the lipoprotein metabolism, we compared plasma lipoproteins in mice lacking LRP5, apolipoprotein E (apoE), or both (apoE;LRP5 double knockout). On a normal chow diet, the apoE;LRP5 double knockout mice (older than 4 months of age) had ~60% higher plasma cholesterol levels compared with the age-matched apoE knockout mice. In contrast, LRP5 deficiency alone had no significant effects on the plasma cholesterol levels. High performance liquid chromatography analysis of plasma lipoproteins revealed that cholesterol levels in the very low density lipoprotein and low density lipoprotein fractions were markedly increased in the apoE;LRP5 double knockout mice. There were no apparent differences in the pattern of apoproteins between the apoE knockout mice and the apoE; LRP5 double knockout mice. The plasma clearance of intragastrically loaded triglyceride was markedly impaired by LRP5 deficiency. The atherosclerotic lesions of the apoE;LRP5 double knockout mice aged 6 months were ~3-fold greater than those in the age-matched apoE-knockout mice. Furthermore, histological examination revealed highly advanced arthrosclerosis, with remarkable accumulation of foam cells and destruction of the internal elastic lamina in the apoE;LRP5 double knockout mice. These data suggest that LRP5 mediates both apoE-dependent and apoE-independent catabolism of plasma lipoproteins.

Genetic defects in the catabolism of plasma lipoproteins are important causes of hypercholesterolemia and atherosclerosis in humans. The prototypic diseases are familial hypercholesterolemia, caused by a defect in the LDL[1] receptor (LDLR) (1), and familial type III hyperlipoproteinemia, caused by a defect in one of the ligands for LDLR, apolipoprotein E (apoE) (2).

ApoE is hypothesized to mediate lipoprotein clearance by binding two receptors: (i) LDLR and (ii) a hepatic chylomicron remnant receptor. ApoE-deficient mice (3–5) and LDLR-deficient mice (6) exhibit hypercholesterolemia, but the severity and manifestations differ markedly. On a normal laboratory chow diet, the apoE knockout mice have much more profound hypercholesterolemia and develop spontaneous atherosclerosis (4).

LDL receptor-related protein 5 (LRP5) is a member of the LDL receptor family that are characterized by the presence of cysteine-rich complement type ligand binding domains. LRP5 binds apoE-containing lipoproteins *in vitro* and is widely expressed in many tissues including hepatocytes, adrenal gland, and pancreas (7).

LRP5 and its homologue, LRP6, are postulated to play as co-receptors for Wnt receptors, Frizzled (8–13). The Wnt signaling pathway plays an essential role in embryonic development (14, 15) and oncogenesis (16) through various signaling molecules including Frizzled receptors (17), LRP5 and LRP6 (8–13), and Dickkopf proteins (11, 12, 18). The Wnt signaling is also involved in adipogenesis by negatively regulating adipogenic transcription factors (19). Recent studies have revealed that loss of function mutations in the LRP5 gene cause the autosomal recessive disorder osteoporosis-pseudoglioma syndrome (20). Consistent with human osteoporosis-pseudoglioma syndrome, LRP5 knockout mice generated by Kato *et al.* (21) exhibit a severe low bone mass phenotype.

Recently, we demonstrated that LRP5-deficient mice develop high plasma cholesterol levels after feeding a high fat diet (22).

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[1] The abbreviations used are: LDL, low density lipoprotein; LDLR, LDL receptor; apoE, apolipoprotein E; CM, chylomicron; Dkk, Dickkopf; HDL, high density lipoprotein; HPLC, high performance liquid chromatography; LRP, LDL receptor-related protein; VLDL, very low density lipoprotein, EMSE, *N*-ethyl-*N*-(3-methylphenyl)-*N*′-succinylethylendiamine; MOPS, 4-morpholinepropanesulfonic acid; PIPES, 1,4-piperazinediethanesulfonic acid.

The hepatic clearance of apoE-rich chylomicron remnants was also markedly decreased in the LRP5 knockout mice. These data suggested that LRP5 plays a role in the hepatic clearance of chylomicron remnants. In addition, we showed that the LRP5-deficient mice fed a normal diet showed marked impaired glucose tolerance. The LRP5-deficient islets had a marked reduction in the levels of intracellular ATP and Ca^{2+} in response to glucose; thereby, glucose-induced insulin secretion was decreased (22). Together with the roles of LRP5 in the bone accrual development (20, 23, 24) as well as in the Wnt signaling pathways (8–11, 13), our data indicated that LRP5 is a multifunctional receptor physiologically linked to common human disorders, including hypercholesterolemia and impaired glucose tolerance.

To further define the role of LRP5 in lipoprotein metabolism, we produced double knockout mice that are deficient in apoE as well as in LRP5 (apoE;LRP5 double knockout mice). In the current paper, we describe that superimposition of an LRP5 deficiency onto apoE deficiency increased plasma cholesterol beyond the level observed with apoE deficiency alone. We also show that fat tolerance was markedly impaired in the LRP5 knockout mice as well as in the apoE;LRP5 double knockout mice. Consistent with extreme hypercholesterolemia, severe atherosclerosis developed in the apoE;LRP5 double knockout mice. These results provide further evidence for the role of LRP5 in the catabolism of plasma lipoproteins.

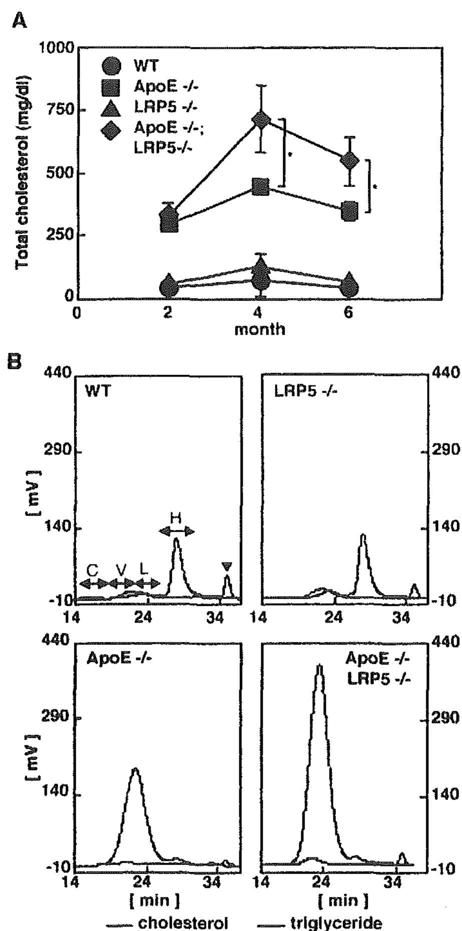

FIG. 1. **Age-dependent changes in plasma cholesterol concentrations in mice with different genotypes fed a normal diet.** *A*, plasma levels of total cholesterol of mice of each genotype at the indicated age were determined enzymatically after 4 h of fasting. Data are mean ± S.D. of six mice. *, $p < 0.01$; Student's *t* test. *B*, HPLC analysis of plasma lipoproteins. Plasma samples from mice of each genotype at 4 months of age were separated by HPLC, and cholesterol (*red line*) and triglyceride (*blue line*) contents were determined as described under "Experimental Procedures." Representative data from six animals with the indicated genotype is shown. The CM, VLDL, LDL, and HDL fractions are labeled *C*, *V*, *L*, and *H*, respectively. Free glycerol is indicated by an *arrowhead*. The cholesterol levels in the CM, VLDL, LDL, and HDL fractions are shown in Table I.

EXPERIMENTAL PROCEDURES

Materials—For the lipoprotein analysis, blood was collected from the retroorbital plexus after 4 h of fasting. Plasma total cholesterol levels were determined in individual mice at each time point by enzymatic assay kits (Wako Pure Chemicals Co., Osaka, Japan).

For the detection of cholesterol and triglycerides with the high performance liquid chromatography (HPLC) method (see below), we obtained enzymatic reagents from Kyowa Medex Co. (Tokyo, Japan). The

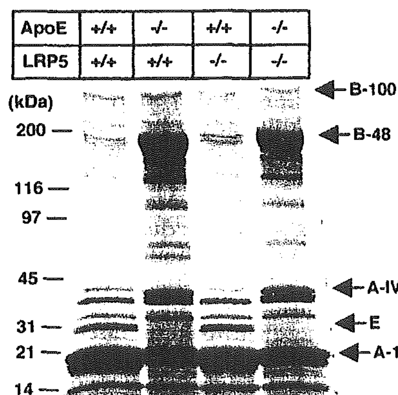

FIG. 2. **SDS-polyacrylamide gel electrophoresis of total lipoprotein fractions.** Equal volumes (1 ml) of plasma were pooled from four mice of different genotypes fed a normal diet and total lipoprotein fractions ($d < 1.215$ g/ml) were isolated by ultracentrifugation, and the delipidated apoproteins were subjected to electrophoresis on an SDS/5–15% polyacrylamide gradient gel. Proteins were stained with Coomassie Blue. Positions of migration of apoB100, apoB48, apoA-VI, apoE, and apoA1 are denoted. Representative data from four independent experiments is shown.

TABLE I
Plasma cholesterol profiles in mice with different genotypes

Plasma samples from mice of each genotype at 4 months of age were separated by HPLC, and cholesterol contents were determined as described under "Experimental Procedures." Values are mean ± S.D. of six mice.

Genotype	Cholesterol			
	CM	VLDL	LDL	HDL
		mg/dl		
ApoE+/+;LRP5+/+	0.10 ± 0.12	2.26 ± 0.28	4.59 ± 1.05	39.9 ± 2.8
ApoE+/+;LRP5−/−	0.03 ± 0.02	3.58 ± 0.40	6.11 ± 1.01	41.2 ± 1.5
ApoE;LRP5+/+	0.16 ± 0.08	180 ± 35[a]	145 ± 7[a]	21.7 ± 3.8[b]
ApoE;LRP5−/−	0.12 ± 0.05	244 ± 24[a, c]	171 ± 21[a, c]	22.9 ± 1.6[b]

[a] $p < 0.01$ *versus* ApoE+/+; LRP5+/+ and ApoE+/+; LRP5−/−.
[b] $p < 0.01$ *versus* ApoE+/+; LRP5+/+ and ApoE+/+; LRP5−/−.
[c] $p < 0.01$ *versus* ApoE−/−; LRP5+/+.

reagent system for cholesterol detection consists of reagent 1 (R1-C) and reagent 2 (R2-C) (R1-C: 20 mM MOPS, pH 7.0, 1.1 mM EMSE, 10 units/ml peroxidase, detergents, and stabilizer; R2-C: 20 mM MOPS, pH 7.0, 1.5 mM 4-aminoantipyrine, 0.68 mM $CaCl_2$, 0.3 units/ml cholesterol esterase, 2 units/ml cholesterol oxidase, 10 units/ml peroxidase, detergents, and stabilizer). The triglyceride reagent system includes reagent 1 (R1-TG) and reagent 2 (R2-TG) (R1-TG: 50 mM PIPES, pH 6.2, 1.1 mM EMSE, 2 mM $MgSO_4$, 4.9 mM ATP, 3 units/ml glycerol kinase, 1.5 units/ml glycerol-3-phosphate oxidase, 5 units/ml peroxidase, detergents, and stabilizer; R2-TG: 50 mM PIPES, pH 6.2, 1.5 mM 4-aminoantipyrine, 2 mM $MgSO_4$, 3 units/ml lipoprotein lipase, 5 units/ml peroxidase, detergents, and stabilizer. Equal amounts of R1 and R2 were mixed before use. After mixing, the cholesterol reagent was used within 4 weeks, and the triglyceride reagent was used within 2 weeks.

Lipoprotein Analysis by a Dual Detection HPLC System—Plasma lipoproteins were analyzed by an improved HPLC analysis according to the procedure as described by Usui *et al.* (25). The HPLC system consisted of an AS-8020 autoinjector, CCPS and CCPM-II pumps, and two UV-8020 detectors (Tosoh, Japan) (26). An SC-8020 system controller (Tosoh) was used for instrument regulation and data collection. Lipoproteins were fractionated on two tandem connected TSKgel LipopropakXL columns (300 × 7.8-mm; Tosoh) with 50 mM Tris acetate, pH 8.0, containing 0.3 M sodium acetate, 0.05% sodium azide, and 0.005% Brij-35 at a flow rate of 0.7 ml/min. The TSK column medium is composed of porous polymermatrices with a nominal bead size of 10 μm and a pore size of 100 nm, which is expected to exclude most of chylomicron (CM) to the void volume. Two TSK columns were connected in tandem and used to obtain higher resolution within a relatively short analytical time. The running buffer was filtered through a 0.22-μm filter (Millipore Corp.) before use and continuously degassed with an SD-8022 on-line degasser (Tosoh) during analysis. The column effluent was split equally into two lines by a Micro-Splitter P-460 (Upchurch Scientific Inc., Oak Harbor, WA), one mixing with cholesterol reagent and the other with triglyceride reagent, in order to achieve simultaneous profiles from a single injection. The two enzymatic reagents were each pumped at a flow rate of 0.35 ml/min for the TSK column. Both enzymatic reactions proceeded at 37 °C in a reactor coil (Teflon, 15 m × 0.4 mm, inner diameter). 10-μl samples diluted with saline were injected by an AS-8020 autoinjector with a presuction volume of 25 μl at intervals of 24 min. The enzymatic determination of cholesterol and triglycerides involved the detection of hydrogen peroxide produced by cholesterol oxidase and glycerol-3-phosphate oxidase, respectively. Total cholesterol and triglyceride concentrations (in mg/dl) were calculated by comparison with total area under the chromatographic curves of a calibration material of known concentration.

SDS-Polyacrylamide Gel Electrophoresis—Total lipoprotein fractions ($d < 1.215$ g/ml) from pooled plasma of the mice were isolated by ultracentrifugation, and the delipidated apolipoproteins were boiled for 3 min in SDS sample buffer containing 2-mercaptoethanol and subjected to electrophoresis on an SDS/5–15% polyacrylamide gel. Proteins were stained with Coomassie Blue.

Fat Tolerance Test—6-month-old male mice were fasted for 16 h, and olive oil (1 ml/30 g body weight; Wako Pure Chemicals Co.) was administered intragastrically as a bolus. Approximately 50 μl of blood was taken from the tail vein at the indicated times for the measurement of triglyceride levels and HPLC analysis.

Mice—LRP5 "knockout" mice (originally C57BL/6J-CBA hybrids (22)), LRP5−/−, have been continually mated with C57BL/6J; N6 and N7 generation descendents from this cross into the C57BL/6J background were used. ApoE−/− mice (3) backcrossed 10 times on the C57BL/6J background were obtained from the Jackson Laboratory (Bar Harbor, ME). To obtain knockout mice that are homozygous for disruption of both the LRP5 and apoE loci, male apoE−/− mice were mated to female LRP5−/− mice. The resulting apoE+/−;LRP5+/− mice were identified by PCR analysis and bred each other to produce apoE−/−; LRP5−/− mice. Experiments were performed with those mice or those with the same genotype from the next generation by breeding apoE−/−;LRP5−/− with each other. Mice were maintained on 12-h dark/12-h light cycles and had free access to a normal laboratory chow diet (4.5% fat, 0% cholesterol, CE-2; CLEA, Tokyo, Japan) and water.

Measurement of Atherosclerotic Lesions—Mice were euthanized, and thoracic and abdominal aorta were used for *en face* staining with Oil Red O to visualize neutral lipid (cholesteryl ester and triglycerides) accumulation. In brief, the aorta was removed, cleaned, and cut open with the luminal surface facing up and then immersion-fixed in 10% formalin in 10 mM phosphate-buffered saline. After rinsing with phosphate-buffered saline, the aorta was thoroughly cleaned of adventitial fat using microforceps and spring iris scissors under a stereoscopic microscope. The inner aortic surface was stained with Oil Red O for 25 min at room temperature. After rinsing with 60% isopropyl alcohol and distilled water, the Oil Red O-stained area was quantified by NIH Image 1.62f software analysis of the digitized microscopic images. Results are expressed as percentage of lipid-accumulating lesion area of the total aortic area analyzed.

For light microscopy, the aortic tissue samples were fixed with 10% formalin in 10 mM phosphate buffer (pH 7.2) and embedded in paraffin. Sections 2–3 μm thick were taken longitudinally through the aortic lumen and stained with hematoxylin and eosin or elastica-Masson. For Oil Red O staining, aortic tissue samples were frozen in OCT compound (Miles Inc., Elkhart, IN). Cryostat tissue sections were cut to a thickness of 5 μm and stained with Oil Red O. Nuclei were counterstained with hematoxylin.

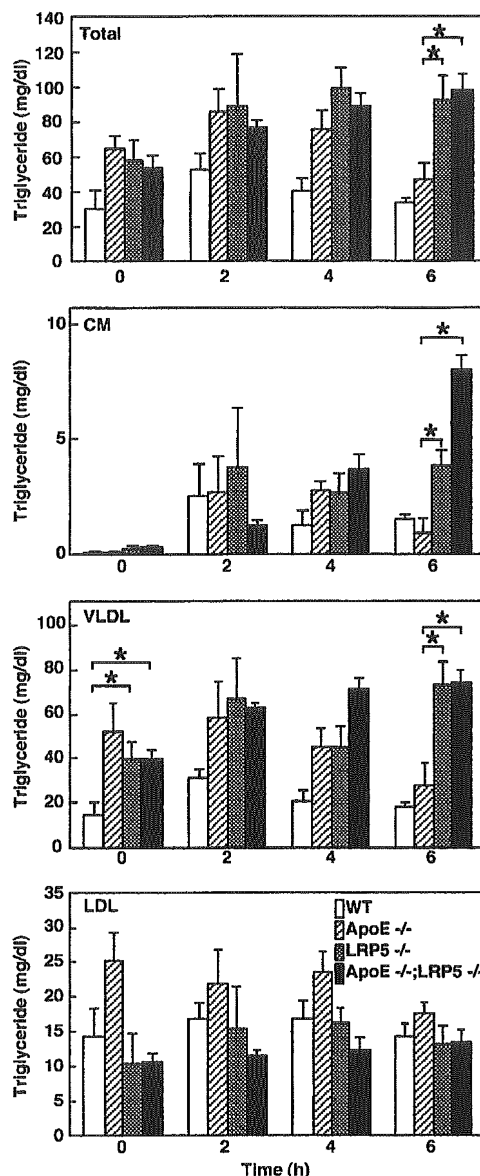

FIG. 3. **Effects of intragastric fat loading on plasma triglyceride levels in mice with different genotypes.** Six males (6 months old) of each genotype received an intragastrically administration of olive oil (1 ml/30 g body weight). At the indicated times, 50 μl of blood was taken from the tail vein and subjected to HPLC analysis. Data are mean ± S.E. of six mice. *, $p < 0.01$; Student's t test.

RESULTS

Plasma Cholesterol and Lipoprotein Profile—Fig. 1 compares the levels of total cholesterol of mice of four different genotypes

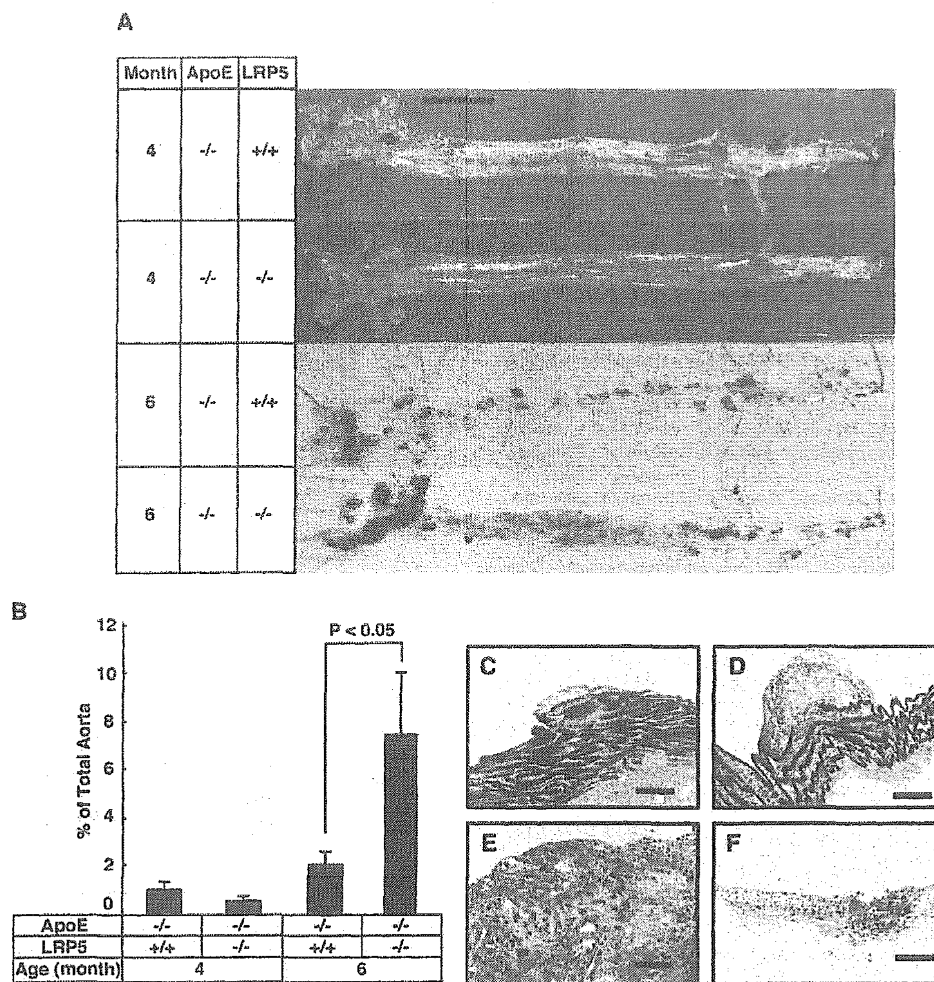

FIG. 4. **Atherosclerotic lesions in apoE and apoE;LRP5 double knockout mice.** *A*, *en face* lipid staining of aortas. Thoracic and abdominal aorta from the indicated genotype was cut open with the luminal surface facing up, and the inner aortic surface was stained with Oil Red O. *Representative data of each genotype are shown. Bar*, 5 mm. *B*, quantitative analysis of *en face* lipid staining. The inner aortic surface area stained with Oil Red O was quantified by NIH Image 1.62f software analysis of the digitized microscopic images. Results are expressed as percentage of lipid-accumulating lesion area of the total aortic area analyzed. Data are mean ± S.D. of six mice. *, $p < 0.01$; Student's *t* test. *C–F*, representative histopathological features of the aorta. *Bars*, 100 μm. *C*, an apoE-knockout mouse (aged 6 months) shows a slightly atheromatous lesion characteristic of the accumulation of foam cells, which is not associated with the destruction of internal elastic lamina (dark brown-colored) or the degenerative change of muscle layer of the aorta (elastica-Masson staining). *D*, one of the multiple atheromatous lesions developed in an apoE;LRP5 double knockout mouse (aged 6 months) manifests a hump structure associated with cholesterin deposits, fibrosis (light green-colored), and elastosis (dark brown-colored). Destruction of the internal elastic lamina adjacent with a degenerative lesion of muscle layer of the aorta is remarkable (elastica-Masson staining). *E*, an atheromatous lesion in an apoE;LRP5 double knockout mouse (aged 6 months) reveals a remarkable accumulation of foam cells, especially marked in the superficial region of atheroma, and a crystal structure of cholesterin deposits (hematoxylin and eosin staining). *F*, an atheromatous lesion in an apoE;LRP5 double knockout mouse (aged 6 months) reveals severe deposition of neutral lipid in the aortic wall, resulting in the destruction of lamellar structure of the elastic fibers (Oil Red O staining).

at the indicated ages. Mice were fed a normal laboratory chow diet containing 4.5% (w/w) fat and 0% cholesterol. Although there were no significant differences in the total plasma cholesterol levels between the apoE knockout mice (apoE−/−; LRP5+/+) and the apoE;LRP5 double knockout mice (apoE−/−;LRP5−/−) at 2 months of age, the cholesterol levels of the double knockout mice older than 4 months were greatly increased (by approximately 60%) beyond the levels observed with apoE deficiency alone. In contrast, LRP5 deficiency alone had no significant effects on the plasma cholesterol levels.

High resolution HPLC analysis (25) of plasma lipoprotein of 4-month-old mice revealed that cholesterol levels in the VLDL and LDL fractions were markedly increased in the apoE;LRP5 double knockout mice compared with the apoE knockout mice (Fig. 1*B* and Table I): the cholesterol levels in the VLDL and LDL fractions in the apoE knockout were 180 ± 35 and 145 ± 7 mg/dl, respectively, and those in the apoE;LRP5 double knockout mice were 244 ± 24 and 171 ± 21 mg/dl, respectively (Table I). There were no significant differences in the levels of CM- and HDL-cholesterol between the apoE knockout mice and the apoE;LRP5 double knockout mice, although HDL-cholesterol levels in these mice were ~50% of those in the LRP5 knockout mice and normal controls. Despite the severe hypercholesterolemia in the apoE knockout and apoE;LRP5 double knockout mice, there were no significant differences in the total triglyceride levels among mice with the four different genotypes (data not shown).

Fig. 2 shows the SDS-polyacrylamide gel electrophoresis of apoproteins in pooled lipoprotein fraction from mice of four different genotypes. Consistent with the previous work by Ishibashi *et al.* (27), the amounts of apoB48 were markedly increased in the apoE knockout mice as well as in the apoE;LRP5 double knockout mice. Despite the severe hypercholesterolemia in the apoE;LRP5 double knockout mice, there were no appar-

ent differences in the pattern of apoproteins between the apoE- and apoE;LRP5 double knockout mice.

Fat Tolerance Test—In a pervious study, we showed that LRP5 plays a role in the hepatic uptake of dietary cholesterol. The LRP5 knockout mice displayed dietary derived hypercholesterolemia due to decreased plasma clearance of chylomicron remnants (22). To further define the role of LRP5, fat tolerance test was carried out using mice of four different genotypes. Mice were fasted for 16 h, and olive oil (1 ml/30 g body weight) was administered intragastrically. As shown in Fig. 3, plasma levels of total triglyceride increased and peaked at about 2 h and then declined toward base line 6 h after loading in both apoE-knockout mice and normal controls. In contrast, the increased levels of plasma triglyceride were sustained for several h after loading in both LRP5 knockout and apoE;LRP5 double knockout mice, indicating that the plasma clearance of intragastrically loaded triglyceride was markedly impaired by LRP5 deficiency. HPLC analysis of plasma lipoproteins revealed that the majority of particles at 6 h after fat loading were in the VLDL fraction.

In addition, we noticed that 16 h of fasting increased the levels of VLDL-triglyceride in the apoE knockout, LRP5 knockout, and apoE;LRP5 double knockout mice. This result may indicate that both apoE and LRP5 mediate the plasma clearance of VLDL-triglyceride induced by fasting.

Atherosclerosis—Aortic atherosclerotic lesions of the apoE knockout and apoE;LRP5 double knockout mice were first analyzed by *en face* lipid staining (Fig. 4*A*). At 4 months of age, the area of the thoracic and abdominal aortas stained by Oil Red O of the apoE;LRP5 double knockout mice was approximately the same as that in the apoE knockout mice. In contrast, at 6 months of age, the lesions in the apoE;LRP5 double knockout mice were ~3-fold larger than those in the apoE knockout mice (Fig. 4*B*).

In histopathology under light microscopic examination, the lesions in the apoE knockout mice at 6 months of age were relatively modest, showing slightly atheromatous lesions with a fatty streak-like structure, which were localized on the surface of aortic intima but were not associated with the destruction of internal elastic lamina or the medial muscle layer (Fig. 4*C*). In contrast, the apoE;LRP5 double knockout mice developed multiple atheromatous lesions manifesting a hump structure, which were associated with cholesterin deposits, fibrosis, and elastosis (Fig. 4*D*). Some of them showed the destruction of internal elastic lamina and the degenerative change of medial muscle layers of the aorta (Fig. 4*E*). In these lesions, severe deposition of neutral lipid was observed (Fig. 4*F*).

DISCUSSION

In the present study, we show extreme hypercholesterolemia in mice lacking both apoE and LRP5. It has been well established that both LDLR and apoE are critical in the plasma clearance of cholesterol-carrying lipoproteins, including LDL and apoE-containing intermediate density lipoprotein and chylomicron remnants (1, 2). In contrast to the mice lacking apoE (3–5) or LDLR (6), the lack of LRP5 alone did not increase the plasma levels of cholesterol on a normal diet, whereas high fat feeding results in hypercholesterolemia in the LRP5 knockout mice (22). Ishibashi *et al.* (27) showed that the plasma cholesterol levels in the double knockout mice lacking both apoE and LDLR were not significantly different from the levels in the apoE knockout mice. The severe hypercholesterolemia developed in the double knockout mice lacking both apoE and LRP5 suggests the presence of an alternative pathway for cholesterol catabolism mediated by LRP5, which appears to be independent of the LDLR pathway.

Consistent with the previous work (22), the LRP5 knockout mice and the apoE;LRP5 double knockout mice displayed markedly impaired fat tolerance. In contrast, the plasma clearance of intragastrically loaded triglyceride was not significantly impaired in the apoE-knockout mice. These observations suggest that LRP5 modulates the plasma clearance of diet-derived triglycerides in the absence of apoE by stimulating the hydrolysis of triglycerides. In this context, it is important to note that LRP5 and LRP6 can bind Dickkopf (Dkk), an antagonist of Wnt proteins (12, 24). Dkk is involved in *Xenopus* head formation and the impaired action of Dkk at LRP5 increases bone density in humans (24). The Dkk sequence consists of two cysteine-rich domains. The C-terminal domain has the typical cysteine pattern of colipase, which is required by pancreatic lipase for the efficient lipid hydrolysis (reviewed in Ref. 28). The C-terminal domain of colipase binds to the C-terminal noncatalytic domain of pancreatic lipase, which is thought to stabilize an active conformation of the lipase, and is also conserved among various lipases, including hepatic and lipoprotein lipases. Detailed sequence analysis and molecular modeling of the Dkk sequence onto the colipase structure suggest that Dkk and colipase have the same disulfide pattern and very similar three-dimensional structures (28). This structural analogy implies a common function (lipid interaction) and raises the possibility that Dkk bound to LRP5 stimulates lipid hydrolysis by interacting with hepatic lipase and/or lipoprotein lipase. Furthermore, the impaired fat tolerance caused by the deficiency of LRP5 may lead to severe hypercholesterolemia in the absence of apoE.

Another explanation for the impaired lipoprotein metabolism in the apoE;LRP5 double knockout mice is that LRP5 may recognize other lipoproteins in addition to apoE-containing lipoproteins. The candidate apoproteins that may be recognized by LRP5 remain unidentified, since the LRP5 deficiency did not significantly alter the pattern of apoproteins in the plasma lipoproteins of the apoE knockout mice or that of normal mice.

In addition to the role of LRP5 in embryonic development and bone development, our current data provide further evidence that LRP5 also plays a role in the metabolism of plasma lipoproteins. Furthermore, consistent with the marked elevation of plasma cholesterol, severe arthrosclerosis developed in the apoE;LRP5 double knockout mice. The remarkable destruction of the internal elastic lamina seen in the lesion of the double knockout mice is characteristic of highly advanced atherosclerosis. The apoE;LRP5 double knockout mice manifesting extreme hypercholesterolemia and highly advanced arthrosclerosis will provide a useful animal model for the research and development of therapeutic agents against hypercholesterolemia and atherosclerosis.

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Expression Cloning and Characterization of a Novel Glycosylphosphatidylinositol-anchored High Density Lipoprotein-binding Protein, GPI-HBP1*

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By expression cloning using fluorescent-labeled high density lipoprotein (HDL), we isolated two clones that conferred the cell surface binding of HDL. Nucleotide sequence of the two clones revealed that one corresponds to scavenger receptor class B, type 1 (SRBI) and the other encoded a novel protein with 228 amino acids. The primary structure of the newly identified HDL-binding protein resembles GPI-anchored proteins consisting of an N-terminal signal sequence, an acidic region with a cluster of aspartate and glutamate residues, an Ly-6 motif highly conserved among the lymphocyte antigen family, and a C-terminal hydrophobic region. This newly identified HDL-binding protein designated GPI-anchored HDL-binding protein 1 (GPI-HBP1), was susceptible to phosphatidylinositol-specific phospholipase C treatment and binds HDL with high affinity (calculated K_d = 2–3 μg/ml). Similar to SRBI, GPI-HBP1 mediates selective lipid uptake but not the protein component of HDL. Among various ligands for SRBI, HDL was most preferentially bound to GPI-HBP1. In contrast to SRBI, GPI-HBP1 lacked HDL-dependent cholesterol efflux. The GPI-HBP1 transcripts were detected with the highest levels in heart and, to a much lesser extent, in lung and liver. *In situ* hybridization revealed the accumulation of GPI-HBP1 transcripts in cardiac muscle cells, hepatic Kupffer cells and sinusoidal endothelium, and bronchial epithelium and alveolar macrophages in the lung.

High density lipoprotein (HDL)[1] plays a key role in the transportation of cholesterol to extrahepatic tissues including steroidogenic tissues and in the reverse transportation of cholesterol from extrahepatic tissues to the liver (1). Unlike the low density lipoprotein (LDL) receptor pathway, the delivery of cholesterol from HDL to cells is mediated by selective lipid uptake from HDL particles and is independent of internalization of HDL. Reverse cholesterol transportation requires the extraction of cholesterol from extrahepatic cells by HDL and the subsequent delivery of cholesterol to hepatocytes.

Several HDL-binding proteins have been identified including class B type I scavenger receptor (SRBI) (2, 3), two candidate hepatic HDL receptors designated HDL-binding proteins 1 and 2 (4, 5), 80- and 130-kDa GPI-anchored HDL-binding proteins expressed in human macrophages (6), 110-kDa GPI-anchored HDL-binding protein expressed in HepG2 cells (7), and recently characterized 95-kDa HDL-binding protein (8). To date, only SRBI appears to be a physiological HDL receptor based on the selective uptake of cholesterol esters into cells and the efflux of cholesterol from cells to HDL mediated by SRBI (1). Consistent with the postulated physiological role, SRBI is highly expressed in tissues that selectively take up cholesterol esters from HDL including liver, adrenal gland, testis, and ovary (3). Although hepatic overexpression of SRBI mediated by an adenovirus encoding SRBI resulted in a dramatic reduction of plasma cholesterol (9), the targeted disruption of the murine *SRBI* gene led to a modest increase in plasma cholesterol (10). This finding may suggest the presence of other HDL receptors that cooperate with SRBI in the metabolism of HDL.

In this study, we identified a novel HDL-binding protein by expression cloning from a murine hepatic cDNA library. This newly identified HDL-binding protein designated GPI-anchored HDL-binding protein 1 (GPI-HBP1) belongs to the GPI-anchored lymphocyte differentiation antigen Ly-6 family, binds HDL with high affinity on the cell surface, and mediates selective lipid uptake from HDL particles. We also describe the ligand specificity and tissue expression of GPI-HBP1.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB095543.

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[1] The abbreviations used are: HDL, high density lipoprotein; LDL, low density lipoprotein; BSA, bovine serum albumin; CHO, Chinese hamster ovary; DiI, 1,1′-dioctadecyl-3,3,3,3′-tetramethylindocarbocyanine perchlorate; GPI, glycosylphosphatidylinositol; HBP1, HDL-binding protein 1; Ly-6, lymphocyte antigen 6; PBS, phosphate-buffered saline; PIPLC, phosphatidylinositol-specific phospholipase C; SRBI, scavenger receptor class B, type I; sHBP1, soluble HDL-binding protein 1.

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