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Triacylglycerol-rich lipoproteins protect lipoprotein lipase from inactivation by ANGPTL3 and ANGPTL4

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ABSTRACT

Lipoprotein lipase (LPL) is important for clearance of triacylglycerols (TG) from plasma both as an enzyme and as a bridging factor between lipoproteins and receptors for endocytosis. The amount of LPL at the luminal side of the capillary endothelium determines to what extent lipids are taken up. Mechanisms to control both the activity of LPL and its transport to the endothelial sites are regulated, but poorly understood. Angiopoietin-like proteins (ANGPTLs) 3 and 4 are potential control proteins for LPL, but plasma concentrations of ANGPTLs do not correlate with plasma TG levels. We investigated the effects of recombinant human N-terminal (NT) ANGPTLs3 and 4 on LPL-mediated bridging of TG-rich lipoproteins to primary mouse hepatocytes and found that the NT-ANGPTLs, in concentrations sufficient to cause inactivation of LPL *in vitro*, were unable to prevent LPL-mediated lipoprotein uptake. We therefore investigated the effects of lipoproteins (chylomicrons, VLDL and LDL) on the inactivation of LPL *in vitro* by NT-ANGPTLs3 and 4 and found that LPL activity was protected by TG-rich lipoproteins. *In vivo*, postprandial TG protected LPL from inactivation by recombinant NT-ANGPTL4 injected to mice. We conclude that lipoprotein-bound LPL is stabilized against inactivation by ANGPTLs. The levels of ANGPTLs found in blood may not be sufficient to overcome this stabilization. Therefore it is likely that the prime site of action of ANGPTLs on LPL is in subendothelial compartments where TG-rich lipoprotein concentration is lower than in blood. This could explain why the plasma levels of TG and ANGPTLs do not correlate.

1. Introduction

Lipoprotein lipase (LPL) has a central role in lipoprotein metabolism through hydrolysis of triacylglycerols (TG) in plasma lipoproteins and as a bridging molecule for receptor mediated endocytosis of lipoproteins and lipoprotein remnants [1–3]. LPL is synthesized in parenchymal cells mainly in adipose tissue, skeletal muscle and heart, but the site of action of LPL is on the luminal side of the capillary endothelium. For a long time LPL was supposed to be bound to endothelial heparan-sulfate proteoglycans (HSPGs) but this view was challenged by the discovery of the glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein 1 (GPIHBP1) as a protein important for binding of LPL to endothelial cells [4]. More recent data show that GPIHBP1 is responsible for transcytosis of LPL over endothelial cells and that this might be its prime function [5,6]. Through mechanisms not yet clearly understood LPL is regulated in a tissue specific manner in response to nutrition and to other physiological factors [7,8]. One example is cold exposure which accelerates LPL activity exclusively in brown adipose tissue to boost TG turnover and channel lipids into BAT for energy expenditure [9]. During the last decade several new factors, among them angiopoietin-like proteins (ANGPTLs) 3 and 4 have emerged as candidates for modulation of the LPL system [10–12]. Injection of ANGPTL3 to mice retarded the removal of TG from plasma and was shown to cause inactivation of LPL [13]. ANGPTL4 inactivates LPL by turning active LPL dimers into inactive monomers [14]. This affects also the bridging function of LPL, which is dependent on the dimeric structure of the enzyme because the binding sites for lipoproteins and receptors partly overlap [15]. Mice overexpressing ANGPTL3 or ANGPTL4 have hypertriglyceridemia, and inactivation of either of the genes in mice results in hypotriglyceridemia [10,16,17]. Genetic variation in *ANGPTL3* or *ANGPTL4* is associated with plasma

Abbreviations: apo, apolipoprotein; BSA, bovine serum albumin; ccd, coiled-coil domain; DMEM, Dulbecco's modified eagle medium; GPIHBP1, glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein 1; HDL, high density lipoprotein; HSPG, heparin sulfate proteoglycan; LDL, low density lipoprotein; LDL-R, LDL-receptor; LPDS, lipoprotein deficient serum; LPL, lipoprotein lipase; LRP1, LDL receptor-related protein 1; PBS, phosphate buffered saline; SPR, surface plasmon resonance; TG, triacylglycerol; TRLs, triacylglycerol rich lipoproteins; VLDL, very low density lipoprotein

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TG levels, demonstrating the importance of ANGPTLs also in humans [18].

ANGPTL3 and ANGPTL4 are both found in blood. In humans average concentrations of approximately 400 ng/ml and 20 ng/ml, respectively, have been reported [19]. Most investigators have failed to find a correlation between plasma ANGPTL3 or 4 levels and plasma TG levels [19–21]. *ANGPTL3* is expressed solely in the liver and is regulated by the liver X receptor [22]. *ANGPTL4* is expressed mainly in the liver and the adipose tissue and is regulated by peroxisome proliferator-activated receptors (PPAR) [23]. Both ANGPTL3 and ANGPTL4 are to some extent cleaved into an N-terminal (NT) coiled-coil domain (ccd) responsible for LPL inhibition and a C-terminal fibrinogen-like domain (fld) with no apparent effect on lipid metabolism; both proteins form oligomers *in vivo* [24–26]. As extensively reviewed by Mattijssen and Kersten ANGPTLs undergo further post-transcriptional modifications by glycosylation where it in the case of ANGPTL3 blocks cleavage whereas ANGPTL4 is seemingly unaffected [27].

ANGPTL4 has been found to modulate LPL activity by direct interaction with the enzyme [14]. There are several factors that can potentially influence on the ANGPTL–LPL interaction. Like LPL, ANGPTL4 binds to heparin [14], heparan sulfate proteoglycans (HSPG) [28] and to extracellular matrix (ECM) components [29]. Heparin protects LPL from inactivation by ANGPTL4 [14,30]. Binding of LPL to GPIHBP1 protects the lipase from inactivation by ANGPTL3 and ANGPTL4 [30].

Here we have investigated if lipoproteins, more specifically chylomicrons, very low density lipoproteins (VLDLs) and low density lipoprotein (LDL) can influence on the inactivation of LPL by recombinant human NT-ANGPTLs3 and 4 *in vitro*. We only investigated the NT-domains of ANGPTLs3 and 4 as they are naturally occurring and the responsible domains for LPL inhibition. We also investigated if NT-ANGPTL3 and/or 4 can influence on LPL-dependent bridging to cells by studies of lipoprotein binding/endocytosis in primary murine hepatocytes. By injection of NT-ANGPTL4 to mice we studied the effects of post-prandial TG levels on inactivation of LPL by NT-ANGPTL4 *in vivo*.

2. Experimental procedures

2.1. Lipoprotein and protein preparation

Expression ready *Escherichia. coli* clones for NT-ANGPTL3 (residues 17–223, accession #NP_055310, pET28a) and NT-ANGPTL-4 (residues 26–229, accession #NP_647475, pET29a) containing the ccd, and part of the fld, with hexa-His tags in the C-terminal end were purchased from Bioclone Inc (CA, USA). Recombinant proteins were expressed in *E. coli* BL21 (DE3) and purified over HisPur Cobalt Resin (Pierce). For this, bacterial pellets were lysed using 50 mM Na-phosphate, 0.3 M NaCl, 6 M guanidinium chloride, 5 mM imidazole, pH 7.4 and frozen at −80 °C. Purification was made according to the manufacturer's instructions under denaturing conditions. The eluted proteins were dialyzed against 20 mM acetic acid and kept as stock solutions at −80 °C. Eukaryotic NT-ANGPTL4 was expressed in 293 T cells as described elsewhere [14]. Immediately prior to animal experiments the NT-ANGPTL4 was dialyzed against phosphate-buffered saline (PBS) pH 7.4. Protein concentrations were determined by the BCA kit (Pierce, Rockford, IL, USA). Preparations of both NT-ANGPTLs showed one major band with the expected molecular mass when run on SDS-PAGE under reducing conditions. Endotoxin levels were measured in the NT-ANGPTL4 preparation using a Limulus Amebocyte Lysate (LAL) kit (Lonza, Walkersville, MD, USA). The solution was found to be essentially endotoxin free (<1 EU/ml) in the dilution used for the *in vivo* experiments. Control mice were injected with PBS containing lipopolysaccharide (Lonza) 23 EU/ml or PBS only. Human apolipoprotein CIII (apoCIII) was expressed in *E. coli* as described [31].

For cell experiments, chylomicron-like TG rich lipoproteins (TRLs) from an apoCII-deficient patient were isolated and radiolabeled as described [32]. VLDL and LDL were isolated from plasma or serum from normal human blood donors through centrifugation using a Beckman L-90 ultracentrifuge and a SW-60 rotor run at 50,000 rpm for 15 h for each step. VLDL was floated at d = 1.006 g/ml. LDL was then collected after a run at d = 1.063 g/ml. The density was adjusted with potassium bromide (KBr) according to the Radding–Steinberg formula. Fetal calf lipoprotein-deficient serum (LPDS) and human LPDS were prepared by removal of all floating lipoproteins after centrifugation at d = 1.25 g/ml for 48 h. All isolated lipoproteins and sera used were dialyzed against PBS in order to remove KBr. Rat intestinal lymph chylomicrons were isolated through cannulation of the thoracic duct as described elsewhere [33]. Lipoproteins were biotinylated as described [34]. Bovine LPL was prepared from milk [35].

2.2. Surface plasmon resonance

Binding studies were performed on a Biacore 2000 (Biacore, Uppsala, Sweden) using CM5 sensor chips. Proteins were immobilized using the Biacore amine coupling kit according to the manufacturer's instructions. PBS pH 7.4 was used as running buffer. The temperature was 25 °C, and the flow was maintained at 30 μl/min. Reference flow cells containing the corresponding amount of covalently bound protein in the form of bovine serum albumin (Sigma) were used as control. Sensorgrams were analyzed using the BIAevaluation software version 3.2 (Biacore, Uppsala, Sweden).

2.3. Preparation of murine primary hepatocytes and studies of lipoprotein uptake

Primary hepatocytes were prepared based on the description by Meredith [36]. In brief, mice were sedated and the portal vein was cannulated. Major vessels caudal to the liver were incised and the liver was perfused (5–8 ml/min) with EDTA-containing perfusion buffer for 1 h. The liver was then removed and disrupted by scissors and forceps in plating medium. After filtration through a 70 μm mesh filter (BD Biosciences), and pelleting by mild centrifugation, the cells were centrifuged once more in a Percoll gradient to obtain pure hepatocytes. Isolated cells were seeded (200×10^3 cells per well) and grown for 4 h at 37 °C, 5% CO_2 in DMEM with 10% LPDS on 12-well collagen I coated plates (Becton Dickinson) before experiments were initiated. Then the cells were incubated with ^{125}I-labeled TRL, with or without 0.6 μg bovine LPL/ml, and with or without NT-ANGPTL3 or NT-ANGPTL4 (molar ratio LPL:NT-ANGPTL3 was 1:6 and LPL:NT-ANGPTL4 was 1:3 calculated on the monomer molecular weight of LPL). Before uptake was measured, the cells were washed three times with PBS containing 100 U heparin/ml. Cells were released from the wells by collagenase and trypsin treatment and were washed with heparin-PBS and spun in a table top centrifuge three times. Finally the cells were lysed and their radioactivity was counted and plotted against cellular protein mass as determined by the modified Lowry method [37]. In some experiments, LPL was pre-incubated in culture medium with NT-ANGPTL3 or NT-ANGPTL4 for 15 min at 37 °C before addition to the cells. The LPL inhibitor tetrahydrolipstatin (THL, Sigma) was used at a concentration of 50 μg/ml and added to cell media prior to addition of LPL.

2.4. Preincubation of LPL with NT-ANGPTLs and chylomicrons, VLDL or LDL

LPL (6.4 nM [14], calculated on monomer molecular weight) was pre-incubated at 25 °C in 50% human serum or PBS, pH 7.4, containing 1% (w/v) BSA with chylomicrons, VLDL or LDL and different concentrations of NT-ANGPTL3 or NT-ANGPTL4 (ranging in molar ratio LPL to NT-ANGPTL from 1:0.25 up to 1:14). After 45 min on an orbital shaker (600 rpm/min), 15 μl samples were taken for analyses of remaining LPL activity in an assay system with a radiolabeled emulsion of soybean