Fatty Acids Bind Tightly to the N-terminal Domain of Angiopoietin-like Protein 4 and Modulate Its Interaction with Lipoprotein Lipase*§

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Background: Angiopoietin-like protein 4 regulates plasma triglyceride metabolism by modulating the activity of lipoprotein lipase.

Results: Fatty acids bind tightly to the N-terminal domain of angiopoietin-like protein 4 and reduce its effect on lipoprotein lipase.

Conclusion: Fatty acids play a role in modulating the effects of angiopoietin-like protein 4.

Significance: A novel mechanism for regulation of the action of angiopoietin-like protein 4 is proposed.

Angiopoietin-like protein 4 (Angptl4), a potent regulator of plasma triglyceride metabolism, binds to lipoprotein lipase (LPL) through its N-terminal coiled-coil domain (ccd-Angptl4) inducing dissociation of the dimeric enzyme to inactive monomers. In this study, we demonstrate that fatty acids reduce the inactivation of LPL by Angptl4. This was the case both with ccd-Angptl4 and full-length Angptl4, and the effect was seen in human plasma or in the presence of albumin. The effect decreased in the sequence oleic acid > palmitic acid > myristic acid > linoleic acid > linolenic acid. Surface plasmon resonance, isothermal titration calorimetry, fluorescence, and chromatography measurements revealed that fatty acids bind with high affinity to ccd-Angptl4. The interactions were characterized by fast association and slow dissociation rates, indicating formation of stable complexes. The highest affinity for ccd-Angptl4 was detected for oleic acid with a subnanomolar equilibrium dissociation constant (Kd). The Kd values for palmitic and myristic acid were in the nanomolar range. Linoleic and linolenic acid bound with much lower affinity. On binding of fatty acids, ccd-Angptl4 underwent conformational changes resulting in a decreased helical content, weakened structural stability, dissociation of oligomers, and altered fluorescence properties of the Trp-38 residue that is located close to the putative LPL-binding region. Based on these results, we propose that fatty acids play an important role in modulating the effects of Angptl4.

Several recent studies indicate that an increased level of plasma triglycerides is an independent and important risk factor for development of atherosclerosis (1–4). Angiopoietin-like protein 4 (Angptl4) is a potent regulator of plasma triglyceride metabolism (5–7). Detailed understanding of how Angptl4 exerts its mechanism may provide options for lowering plasma triglyceride level. Both in vitro and in vivo experiments suggest that Angptl4 reduces the activity of lipoprotein lipase (LPL), a key enzyme in plasma triglyceride metabolism (5–7). LPL is mainly synthesized in adipocytes and myocytes. After secretion, the enzyme finds its way through the extracellular matrix to the luminal side of the capillary endothelium (8, 9). The inactivation of LPL occurs through a unique mechanism. Angptl4 acts as an extracellular unfolding molecular chaperone on LPL, converting the active dimeric enzyme into inactive monomers (10). In addition to effects on plasma triglyceride metabolism, Angptl4 is involved in the regulation of intracellular lipolysis in adipose tissue (5), glucose metabolism (11), angiogenesis (12, 13), cancer development (14), and wound healing (15).

Angptl4 is a 50-kDa, glycosylated secretory protein that belongs to the angiopoietin-like protein family (16, 17). Based on structure predictions, angiopoietin-like proteins are composed of two distinct structural units, an N-terminal coiled-coil domain and a C-terminal fibrinogen-like domain. In plasma and in cell cultures, proteases cleave Angptl4 into fragments that correspond to the N-terminal (ccd-Angptl4) and C-terminal folding domains. Both full-length and cleaved Angptl4s are present in human plasma (18–20). ccd-Angptl4 tends to form oligomers through intermolecular disulfide bonds between the cysteines at positions 76 and 80. Replacement of these residues prevents oligomerization of the protein and hinders its ability to increase plasma triglycerides in rats (17, 20) and mice (21), indicating physiological importance of the disulfide bonds.

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The abbreviations used are: Angptl4, angiopoietin-like protein 4; hAngptl4, human angiopoietin-like protein 4 expressed in yeast; mAngptl4, human angiopoietin-like protein 4 expressed in mouse myeloma cells; ccd-Angptl4, coiled-coil domain of Angptl4; hccd-Angptl4, human coiled-coil domain of Angptl4; mccd-Angptl4, mouse coiled-coil domain of Angptl4; LPL, lipoprotein lipase; ITC, isothermal titration calorimetry; SPR, surface plasmon resonance; DGG, 1,2-D-3,6-diacylglucero-3,5-diacylglycerol; 6′-methyl-resorufin) ester; pNPP, p-nitrophenyl butyrate; BisTris, 2-[bis(2-hydroxyethyl)aminio]-2-(hydroxymethyl)propane-1,3-diol.

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Recombinant ccd-Angptl4 produced by mammalian cell systems are usually oligomers linked via disulfide bonds, although bacterial cells produce the protein without intermolecular disulfides (22).

The two domains of Angptl4 have distinct roles. The N-terminal domain of Angptl4 binds to LPL with high affinity, resulting in inactivation of the enzyme (10, 23). It has been proposed that the LPL-binding region in Angptl4 is located between residues 38 and 52 (24, 25). This is supported by population-based studies that indicate that individuals carrying the E40K variant of Angptl4 have low plasma triglyceride levels (26). Furthermore, a synthetic peptide spanning residues 44–55 of human Angptl4 was able to inhibit LPL, but the affinity of this peptide for LPL was much lower than that of full-length ccd-Angptl4 (25).

Like LPL, the N-terminal domain of Angptl4 exhibits high affinity for negatively charged cell surface components, such as heparan sulfate and dermatan sulfate, and for negatively charged components of the connective tissue (11). This property might be important for the physiological modulation of LPL activity by Angptl4 during transport of the enzyme from its sites of synthesis in adipocytes and myocytes to its sites of action on the luminal side of the capillary endothelium. Recently, it was demonstrated that binding of Angptl4 to a cell surface is necessary for the specific cleavage of the protein into the two domains (23).

Both LPL and Angptl4 are highly expressed in adipose tissue, at least in rats and mice. Regulation of LPL activity in rat adipose tissue is rapid and mainly due to changes in the ratio of active LPL dimers to inactive LPL monomers, without affecting the total mass of the enzyme (27, 28). It is likely that Angptl4 plays an important role in this conversion. During the fed to fasted transitions, changes in Angptl4 mRNA abundance are inversely correlated to LPL activity in adipose tissue (10). These data demonstrate that Angptl4 expression responds rapidly to fasting, and Angptl4 expression is up-regulated by activated peroxisome proliferator-activated receptors α, δ, and γ (29–33).

The plasma concentrations of Angptl4 in humans are low. There are large individual variations ranging from 2 to 158 ng/ml (34, 35). Size exclusion chromatography of plasma demonstrated both full-length and cleaved Angptl4 eluting together with lipoproteins, suggesting direct interaction of Angptl4 with lipoproteins (36). Several studies have failed to demonstrate any direct association between plasma levels of Angptl4 and plasma triglyceride levels (35, 36). One possible explanation is that glycosylphosphatidylinositol-anchored high density lipoprotein-binding protein (GPIHBP-1), a recently identified LPL-anchoring protein produced by endothelial cells, protects LPL from the inhibitory effect of Angptl4 (37). Furthermore, triglyceride-rich lipoproteins that interact avidly with LPL tend to diminish the inhibitory effect of Angptl4 (38). These observations suggest that the interaction of Angptl4 with LPL is influenced by factors in plasma or that it occurs before LPL has reached the luminal side of the endothelium.

We have examined whether free fatty acid can affect structure/function properties of recombinant variants of Angptl4s and the inhibitory action of ccd-Angptl4 on LPL. It was previously shown that fatty acids bind to LPL, stabilize the enzyme, and cause strong product inhibition of LPL activity (39). However, it was unknown whether fatty acids can influence the Angptl4/LPL interaction. Our results show that fatty acids reduce the inhibitory effect of Angptl4 on LPL. Surface plasmon resonance, isothermal titration calorimetry, and fluorescence measurements demonstrate that fatty acids bind tightly to ccd-Angptl4, close to the putative LPL-binding site. Upon binding of fatty acids, ccd-Angptl4 undergoes conformational changes resulting in decreased helical content, weakened structural stability, and changes in fluorescence properties. Based on these results, we propose that fatty acids play an important role in modulating the effects of ccd-Angptl4.

EXPERIMENTAL PROCEDURES

Reagents—Bovine LPL was purified from milk and was stored as a stock solution of about 0.5 mg/ml (200 units/ml) in 1 M NaCl, 10 mM BisTris buffer, pH 6.5 (40). Heparin was obtained from Lövens (Malmö, Sweden). For expression of human ccd-Angptl4 (hccd-Angptl4), residues 26–184 with a glutamic acid and a hexa-His tag at the C-terminal end and a methionine at the N-terminal end were incorporated into vector pET29a, expressed in Escherichia coli BL21 (DE3), and purified on a cobalt-chelate-agarose column (Pierce HisPur cobalt resin). Mouse ccd-Angptl4 (mcdd-Angptl4) was produced and purified as described (10). Full-length human Angptl4 (hAngptl4) was expressed in Pichia pastoris X-33 using the PPICZα vector. Recombinant protein was purified on a nickel-Sepharose column (HiTrap, GE Healthcare). Based on SDS-PAGE under reduced conditions, all three recombinant proteins were essentially pure, and their bands corresponded to the expected molecular weights. Fatty acids were obtained from Cayman Chemical Co. p-Nitrophenyl butyrate (pNPB), 1,2-O-dilauryl-

Inhibition Kinetics—Inhibition kinetics of LPL during hydrolysis of pNPB was measured in 20 mM Hepes, 0.15 M NaCl, pH 7.4, at 20 °C. The experiments were performed in the presence of 1 IU heparin/ml to stabilize the enzyme. In these exper-
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injected to the flow cells at concentration of 0.1 μM for 700 s. Binding experiments with fatty acids and SDS were conducted at 25 °C in 20 mM HEPES, 0.15 M NaCl, pH 7.4, 0.15 M NaCl containing 0.1% ethanol (running buffer). A part of binding experiments were performed in lipoprotein-free human plasma that was diluted 1:10 (v/v) in the running buffer. To study the LPL/ccd-Angptl4 interaction, mccd-Angptl4 in the absence or presence of various concentration of oleic acid was injected into the sensorchip loaded with biotinylated LPL to a surface concentration of 4000–5000 response units. Conditions were as above but with 0.1% ethanol.

**ITC Measurements**—Experiments on the interaction of ccd-Angptl4 and fatty acids were carried out at 25 °C using a Nano ITC200 isothermal titration calorimeter (TA Instruments Water-LLC, New Castle, DE). Purified hccd-Angptl4 was dialyzed against 20 mM HEPES buffer, pH 7.4. Stock solutions of fatty acid in ethanol were diluted into the same buffer. A solution of 10 μM oleic acid or myristic acid together with 1.5 μM BSA was loaded to the ITC sample cell (1.0 ml), and a solution of 85 μM hccd-Angptl4 was placed in the injection syringe (250 μl). A titration experiment consisted of 20 consecutive injections of 10-μl volumes, each with a 5-min interval between injections. Dilution heat was measured by injecting hccd-Angptl4 into buffer with 1.5 μM BSA and was subtracted from the experimental curves prior to data analysis. Values for the standard molar enthalpy change for the binding ΔH, the binding constant Kp, and the binding stoichiometry n, were obtained using Equation 2,

\[ 
\Delta \sum Q = 0.5 \cdot \Delta H \cdot V \cdot (n \cdot [C] + [A]) + K_d - \sqrt{(n \cdot [C] + [A] + K_d)^2 + 4 \cdot n \cdot [C] \cdot [A]} \]  

(Eq. 2)

where \( \Delta \sum Q \) is the cumulative heat determined after each hccd-Angptl4 addition; V is the sample cell volume; [C] is the total concentration of fatty acid; [A] is the concentration of hccd-Angptl4 after each addition; n is the number of fatty acids bound per hccd-Angptl4 molecule, and \( K_d \) is the equilibrium dissociation constant.

**Fluorescence Quenching Experiments**—Tryptophan quenching experiments were carried out with 0.5 μM mccd-Angptl4, hccd-Angptl4, or L-tryptophan at acrylamide concentrations ranging from 0 to 0.7 M in 20 mM Hepes, pH 7.4, 0.15 M NaCl at 25 °C with or without 0.4 μM oleic acid. Tryptophans were excited at 280 nm, and the resulting emission was recorded at 342 nm. The measurements were performed on a Shimadzu RF-5301 fluorescence spectrophotometer. Quenching of L-tryptophan was analyzed by the Stern-Volmer Equation 3 (43),

\[ \frac{F_0}{F} = 1 + K_{SV}[Y] \]  

(Eq. 3)

where \( F_0 \) and F are fluorescence intensities before and after the addition of acrylamide; \( K_{SV} \) is the effective quenching constant, and [Y] is acrylamide concentration. Quenching data for mccd-Angptl4 and hccd-Angptl4 were analyzed by Equation 4 (38),

\[ F = \frac{F_{0a}}{1 + K_{SV}[Y]} + F_{ob} \]  

(Eq. 4)

where \( F_{0a} \) and \( F_{ob} \) are the fluorescence intensities before and after the addition of acrylamide, respectively.
where $F$ is the fluorescence intensity after the addition of acrylamide; $F_{ac}$ is the intensity of acrylamide-accessible fraction of ccd-Angptl4 in the absence of acrylamide, and $F_{ab}$ is the intensity of fluorescence of the fraction that is not quenched by acrylamide. The total fluorescence in the absence of acrylamide ($F_0$) is given by $F_0 = F_{ac} + F_{ab}$. Fluorescence emission spectra measurements are described in supplemental Methods.

Circular Dichroism Spectroscopy—Circular dichroism spectra were recorded at 20 °C on a Jasco CD spectrophotometer using a 0.1-cm path length quartz cell. The experiments were carried out in 20 mM potassium phosphate buffer, 0.1% ethanol, pH 7.4. The protein concentration in the samples was 0.23 mg/ml. Molar excess of oleic acid over the proteins varied from 100 to 4. Random error and noise were reduced for each spectrum by averaging six scans in the wavelength range 260 to 190 nm. The signal acquired for the buffer used for dilution of the protein was subtracted from the spectra. The CD spectra were recorded at 20 °C on a Jasco CD spectrophotometer.

Limited Proteolysis by Trypsin—Tryptic digestion of hccd-Angptl4 was performed at 25 °C in 0.1 M NaH$_2$PO$_4$, 0.15 M NaCl, pH 7.4, in the presence or absence of stoichiometric amounts of myristic acid. Aliquots were removed from the reaction mixture at 0, 10, 20, 30, 40, 50, and 60 min. PMSF was immediately added to the samples to a final concentration of 1 mM to stop the reaction. The samples were run on SDS-PAGE (16% gels) and stained with Fermentas PageBlue™ protein staining solution. The gels were scanned using a Syngene G:Box gel imaging system.

Oligomeric Distribution of hccd-Angptl4 by Size Exclusion Chromatography—Experiments were conducted at 25 °C on a Sepharose G-75 column (10/300 mm) equilibrated with 20 mM Hapes, pH 7.4, with or without 3.0 μM linoleic acid. The column was first calibrated with dextran blue (2000 kDa), BSA (monomer 66 kDa, dimer 132 kDa), chymotrypsinogen A (25 kDa), myoglobin (16.7 kDa), and dinitrophenyl aspartate (299.5 Da). Subsequently, 2.3 mg of hccd-Angptl4 in 2 ml running buffer was applied to the column. Eluted hccd-Angptl4 was determined by absorbance at 280 nm.

Data Analysis—The SPR data were analyzed by BIASevaluation 3.1 software. The inhibition, ITC, and fluorescence data were analyzed using Sigma Plot 10 software.

RESULTS

The structural/functional characterization of the recombinant Angptl4 variants is presented in supplemental material.

Effects of Fatty Acids on Inhibition of LPL by Angptl4—The effects of fatty acids on the ccd-Angptl4-dependent inhibition of LPL was investigated using the water-soluble substrate pNPB to avoid possible incorporation of fatty acids into substrate micelles or emulsion particles. Moreover, it has been shown that emulsified long chain triglyceride substrates protect LPL from inhibition/inactivation by Angptl4 (38). Hence, the simplicity of the pNPB system allowed us to focus on the effects of fatty acids on the Angptl4/LPL interaction and to examine the kinetics of this interaction in detail. As with DGGG, the ccd-Angptl4 proteins reduced the initial rate of hydrolysis of pNPB by LPL. The inactivation rate constants $k_i$ were the same order of magnitude (10$^{-4}$ s$^{-1}$) in the presence or absence of ccd-Angptl4s, demonstrating inhibition of LPL activity rather than inactivation (Fig. 1A). Fatty acids reduced the inhibition of LPL activity by ccd-Angptl4 but influenced less the inactivation kinetics. Data for palmitic acid are presented in Fig. 1A. Already submicromolar concentrations of palmitic acid protected LPL from the inhibitory effect of hccd-Angptl4 (Fig. 1A, compare lines 6–8 with line 5). At the same time, palmitic acid did not influence the initial activity of LPL (Fig. 1A, compare lines 1–4).

In Fig. 1B, the relative LPL activities are expressed as ratios of activities (ratios of $v$ values, see Equation 1) in the presence of...
ccd-Angptl4, with or without different fatty acids, to the activity of the enzyme without any additions. Oleic acid exhibited the highest ability to prevent inhibition of LPL activity by ccd-Angptl4, possibly by preventing formation of ccd-Angptl4/LPL complexes. At a concentration 0.5 μM, oleic acid increased the relative activity from 20 to 55% (Fig. 1A). Palmitic acid and myristic acid were less efficient, increasing the relative activity to 51 and 33%, respectively, at a concentration 1 μM (Fig. 1B). Linoleic and linolenic acid up to a concentration 3 μM were unable to protect LPL against the inhibitory effect of hccd-Angptl4 (Fig. 1B).

In the next experiments, LPL was preincubated with hccd-Angptl4 and fatty acids before activity measurements. Residual LPL activities were determined by a phospholipid-stabilized emulsion of long chain triglycerides (Intralipid) (Fig. 2A), triglyceride-rich lipoproteins (Fig. 2B), or DGGR (supplemental Table 1).

When Intralipid was used as substrate, at least 5 μM oleic acid in the preincubation mixture was needed for stabilization of LPL (Fig. 2A, line LPL), and concentrations higher than 10 μM oleic acid were required for the protection LPL from hccd-Angptl4 (Fig. 2A, line LPL + hccd-Angptl4). An excess of BSA (0.1%, 15 μM) over oleic acid (highest concentration 25 μM) in the preincubation mixture increased the stability of the enzyme (Fig. 2A, line LPL + BSA) but abolished the protective effect of oleic acid on the hccd-Angptl4-mediated inactivation (Fig. 2A, line LPL + BSA + hccd-Angptl4).
To study fatty acid influence on the hccd-Angptl4/LPL interaction under more in vivo-like conditions, preincubation experiments were performed in a phosphate buffer that contained 1.5 or 3% BSA or in lipoprotein-free human plasma. In these experiments, the oleic acid effect on the hccd-Angptl4- or h_{2}Angptl4-induced inactivation of LPL was measured as a function of the oleic acid/albumin molar ratio (Fig. 2B and supplemental Fig. 3). When preincubation was performed in the presence of 1.5 or 3% BSA, the protection effect was not detectable at low oleic acid/BSA ratios but increased steeply when the ratio exceeded 6, reaching the maximal stabilization at a ratio close to 8 (supplemental Fig. 3). Lipoprotein-free human plasma, which itself contained 0.31 mm albumin and 0.31 mm fatty acids, significantly reduced the Angptl4-mediated inactivation of LPL (Fig. 2B). Addition of oleic acid to the lipoprotein-free plasma linearly increased the stability of LPL. Each albumin and fatty acid, significantly reduced the Angptl4-mediated inactivation of LPL (Fig. 2B). Addition of oleic acid to the lipoprotein-free plasma linearly increased the stability of LPL. Each albumin molecule is able to bind seven fatty acids with affinity constants ranging from 10^{7} to 69 \times 10^{7} (44). Hence, the effect was observed already before saturation of albumin with oleic acid. The difference between the results obtained in the presence of BSA (supplemental Fig. 3) compared with those in human plasma (Fig. 2B) suggested that other plasma components than albumin contributed to the protection of LPL from inactivation by ccd-Angptl4.

Chromatography on heparin-Sepharose revealed that oleic acid protected the dimeric LPL from the Angptl4-induced dissociation to monomers (Fig. 2C). When LPL was incubated with hccd-Angptl4 in the presence of oleic acid, it eluted from the column by 1.1–1.3 M NaCl. This elution concentration of NaCl corresponds to the dimeric form of LPL (10). When the incubation was performed without oleic acid, LPL eluted as a monomer by 0.6–0.8 M NaCl (Fig. 2C).

The ability of oleic acid to reduce the hccd-Angptl4 effect on LPL retained when a mixture of hccd-Angptl4 and oleic acid was run on a gel filtration column before incubation with LPL (Fig. 2D). This indicated that oleic acid remained bound to hccd-Angptl4 during the chromatography. In addition, this result suggests that the protection of LPL is likely to be due to the interaction between oleic acid and hccd-Angptl4.

Results obtained by DGGR are presented in supplemental Table 1. After 30 min of incubation in the presence of hccd-Angptl4, LPL lost 46% activity compared with what happened in the absence of hccd-Angptl4. Oleic acid at a concentration of 0.2 or 3.0 \mu M stabilized the enzyme and completely abolished the inactivation by hccd-Angptl4. When both hccd-Angptl4 and oleic acid were present in the incubation mixture, the stability of LPL was in fact increased compared with LPL and oleic acid alone. This suggests formation of an active ternary complex of LPL, fatty acid, and hccd-Angptl4.

Influence of Fatty Acids on Binding of Angptl4 to LPL—To investigate whether fatty acids diminished the inhibitory effect of ccd-Angptl4 on LPL by lowering the affinity between these proteins, SPR experiments were performed. Biotinylated LPL was coupled to immobilized streptavidin and mccd-Angptl4 was injected to the sensorchip in the absence or presence of oleic acid (Fig. 3A). Binding of mccd-Angptl4 to LPL was reduced when the concentration of oleic acid was increased from 0 to 100 nm (Fig. 3A). The sensorgrams presented in Fig. 3A could be fitted to a simple 1:1 binding model, but only if the first 200 s of the dissociation phases were used in the analyses. More complex models provided by BIAevaluation software did not describe the curves better. The estimated association rate constants (k_{a}) were as follows: 5.9 \times 10^{5} M^{-1} s^{-1} in the absence of oleic acid and 3.8 \times 10^{5}, 2.6 \times 10^{5}, and 1.7 \times 10^{5} M^{-1} s^{-1} in the presence of 25, 50, and 100 nm of oleic acid, respectively. The dissociation rate constants (k_{d}) were not significantly influenced by the oleic acid concentration. The values ranged between 1.0 \times 10^{-3} and 1.4 \times 10^{-3} s^{-1}. Separate experiments were performed to find out whether oleic acid influenced the amount of mccd-Angptl4 bound to LPL at equilibrium. As can be seen in the Fig. 3B, no further substantial increase of bound mccd-Angptl4 was detected when the concentration of
oleic acid was higher than 100 nm. This suggests formation of a ternary complex LPL-mccd-Angptl4-oleic acid at the surface of the sensorchip.

Fatty Acids Bind to Angptl4 with High Affinity—Previous studies had demonstrated that fatty acids bind to LPL and stabilize its enzymatic activity (39, 45). Those experiments were done at micellar concentrations of fatty acids (above the critical micellar concentration). The effects of fatty acids on the Angptl4/LPL interaction studied here were detected already at pre-micellar concentrations (below the critical micellar concentration). We therefore investigated whether fatty acids below their micellar concentrations can bind to LPL or ccd-Angptl4. Using SPR, micromolar concentrations of oleic acid were needed to detect binding to immobilized LPL, although all three recombinant variants of Angptl4 bound fatty acids already at nanomolar concentrations. A binding sensorgram of 1.2 μM oleic acid to LPL is presented in Fig. 4A. As can be seen, at the end of injection only few response units of oleic acid were bound to the immobilized LPL. At the same time, significant binding of oleic acid to the Angptl4 variants was observed already when oleic acid concentration was in the nanomolar range. Sensorgrams for binding of 40 nM oleic acid to the Angptl4 variants are presented in Fig. 4, B–D. In all cases, oleic acid did not show measurable dissociation from the Angptl4 variants during the period used in the experiments, indicating strong interaction.

Binding of oleic acid to the immobilized full-length Angptl4 (hAngptl4) was observed even in the presence of human plasma (Fig. 5A). In accordance with the protection experiments in plasma (Fig. 2B), the binding between hAngptl4 and oleic acid was detectable before complete saturation of albumin with oleic acid (Fig. 5A). In addition to oleic acid, SPR experiments were performed with myristic, palmitic, linoleic, and linolenic acid. Binding at 10 nM concentration to immobilized mccd-Angptl4 is presented in Fig. 5B. At this concentration, similar binding was observed for oleic acid and palmitic acid. Binding was also detectable for myristic acid but not for linoleic and linolenic acid. It was not possible to remove the bound fatty acids without affecting the binding properties of the immobilized ccd-Angptl4. Therefore, single cycle experiments were performed with all Angptl4 variants by increasing stepwise the fatty acid concentration in the flow phase without dissociation between injections. For comparison, SPR was used for analysis of binding of oleic acid to albumin. The sensorgrams for binding of oleic acid at 21, 42, 84, 125, 250, and 500 nM are presented in supplemental Fig. 4. As can be seen, this interaction is completely different from the oleic acid/ccd-Angptl4 interaction. Although the association of oleic acid was detectable already at 21 nM oleic acid, the complex was not stable, and oleic acid dissociated rapidly from the immobilized protein.

Response values at equilibrium for binding of oleic acid to immobilized mccd-Angptl4, hcccd-Angptl4, hAngptl4, BSA, and LPL as functions of oleic concentration are shown in Fig. 5C. In the case of the Angptl4 variants, saturation was achieved when the oleic acid concentration was close to 1 μM. However, these curves were not typical binding hyperbolas; they increased steeply but became almost linear when the oleic acid concentration was between 30 and 40 nM and reached saturation when oleic concentration was higher than 1 μM. This suggested that the immobilized Angptl4 variants bound several fatty acids per protein monomer. The response of the bound fatty acids exceeded at least 3–4-fold the level expected for a 1:1 complex. Binding of oleic acid to BSA also occurred in two phases with saturation at 300–400 nM, followed by a continued increase in binding when the oleic acid concentration was further increased. Oleic acid bound to LPL with clearly lower affinity than to other studied proteins. Binding to LPL was detecta-
acids were injected at a concentration of 10 nM. Nonspecific binding to flow
0.15 M NaCl and 0.1% ethanol.

measurements were performed at 25 °C in 20 mM Hepes, pH 7.4, containing
ing of oleic acid at various concentrations was detected at equilibrium. All

The kinetics obtained by using single cycle experiments was
to prevent disturbing and significant heat production due to
dilution or/and nonspecific interactions of hccd-Angptl4 with
surfaces of the ITC cell. Small aliquots of concentrated hccd-
Angptl4 were added to a sample cell containing oleic acid
and BSA. After each addition the heat formation was measured. The
ITC raw data for a titration of 10 M oleic and myristic acid
with hccd-Angptl4 are shown in Fig. 6, A and B. After integration
of the peaks and subtraction of the dilution effect, typical titration
curves were obtained for both fatty acids (Fig. 6, C and D). Corresponding cumulative heat production curves are
displayed in Fig. 6, E and F. A molar ratio 0.4–0.5 of hccd-Angptl4
over oleic or myristic acid was sufficient to complete the titra-
tions of high affinity binding, indicating that each hccd-Angptl4
monomer bound at least two fatty acids with high affinity (Fig.
6, C and D or E and F). The cumulative heat production data
were subjected to least squares analysis to extract estimates of
the affinity constants, stoichiometries, and binding enthalpies.
These calculations were performed using Equation 2. For both
fatty acids, the best fit to the data was obtained when the stoic-
chiometry of oleic acid over hccd-Angptl4 was close to 2.

ble only when the oleic acid concentration exceeded 1 μM, and
saturation was not reached.

The kinetics obtained by using single cycle experiments was
too complex to be described unequivocally with the models
provided by the BIAspecific software. However, at low con-
centrations of fatty acids (less than 40 nM), when the increase of

response units remained lower than 50, binding curves for fatty
acids to the Angptl4 variants followed a simple 1:1 binding
model. Kinetic constants obtained at these low concentrations
are presented in Table 1. As can be seen, all studied fatty acids
bound to the immobilized Angptl4 variants with a reasonably
fast on-rate (kₐ = 10³–10⁴ M⁻¹ s⁻¹) and slow off-rate (kₐ<10⁻⁴
s⁻¹), resulting in a low equilibrium constant Kₐ. A special case
was oleic, which dissociated so slowly that it was not possible to
calculate the kₐ. The calculated kₐ for binding of oleic acid to
BSA is comparable with the kₐ value of the interaction of ccd-
Angptl4 with oleic, palmitic, and myristic acid, but the kₐ value
for the oleic acid/albumin interaction was much higher. For
comparison, we tested the ability of ccd-Angptl4 to bind to
other negatively charged amphipathic compounds like SDS.

The affinity of ccd-Angptl4 for SDS appeared to be much lower
than that for fatty acids, and the calculated equilibrium disso-
ciation constant was several magnitudes higher than those for
fatty acids (Table 1).

The results of the SPR experiments show that although the
Angptl4 variants interacted with all studied fatty acids, oleic
acid bound substantially stronger than the other fatty acids.
This was consistent with the ability of oleic acid to protect LPL
from inhibition/inactivation by Angptl4s (see Fig. 1B).

ITC Measurements—To further characterize the interaction
of hccd-Angptl4 with fatty acids, ITC experiments were carried
out. Because ITC is time- and protein-consuming, the mea-
surements were performed only with oleic and myristic acid.
ITC enabled us to quantitate the stoichiometries and enthalpies
for the binding of fatty acid to hccd-Angptl4.

Reproducible results were obtained only when fatty acids
were titrated with hccd-Angptl4 in the presence of a low con-
centration of BSA (1.5 μM). The presence of BSA was necessary
to prevent disturbing and significant heat production due to
dilution or/and nonspecific interactions of hccd-Angptl4 with
surfaces of the ITC cell. Small aliquots of concentrated hccd-
Angptl4 were added to a sample cell containing oleic acid
and BSA. After each addition the heat formation was measured. The
ITC raw data for a titration of 10 μM oleic and myristic acid
with hccd-Angptl4 are shown in Fig. 6, A and B. After integration
of the peaks and subtraction of the dilution effect, typical titration
curves were obtained for both fatty acids (Fig. 6, C and D).

FIGURE 5. Binding of fatty acids to Angptl4 variants, LPL, and BSA, as
measured by SPR. A, binding of oleic acid to immobilized hAngptl4 (7.2
ng/mm²) at various oleic acid/albumin ratios in human lipoprotein-free
plasma diluted 10 times in 20 mM Hepes, 0.15 M NaCl, pH 7.4. The numbers 6.1,
3.4, and 2.2 indicate the molar excess of oleic acid over albumin. B, interaction
of mccd-Angptl4 (1.7 ng/mm²) with various fatty acids. The different fatty
acids were injected at a concentration of 10 mM. Nonspecific binding to flow
cells without mccd-Angptl4 was subtracted from each curve. C, binding of
oleic acid to mccd-Angptl4, hccd-Angptl4, hAngptl4, LPL, and BSA. The pro-
teins were immobilized to surface concentrations of 80–90 fmol/mm². Bind-
ing of oleic acid at various concentrations was detected at equilibrium. All
measurements were performed at 25 °C in 20 mM Hepes, pH 7.4, containing
0.15 M NaCl and 0.1% ethanol.

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Table 1

Kinetic constants for interactions of recombinant Angptl4 variants and BSA with fatty acids and SDS as determined by SPR analysis

| Protein       | Ligand      | $K_a$ | $K_d$ | $K_d$ |
|---------------|-------------|-------|-------|-------|
| mccd-Angptl4  | Oleic acid  | $3.3 \pm 0.4 \times 10^2$ | $10^{-6}$ | $<3 \mu M$ |
|               | Palmitic acid| $2.9 \pm 0.1 \times 10^2$ | $3.8 \pm 0.2 \times 10^{-5}$ | $0.13 \mu M$ |
|               | Myristic acid| $2.1 \pm 0.2 \times 10^2$ | $2.7 \pm 0.3 \times 10^{-4}$ | $1.3 \mu M$ |
|               | Linoleic acid| $0.5 \pm 0.1 \times 10^1$ | $5.6 \pm 0.1 \times 10^{-4}$ | $112 \mu M$ |
|               | SDSL        | $0.4 \pm 0.1 \times 10^1$ | $2.4 \pm 0.1 \times 10^{-4}$ | $60 \mu M$ |
| hccd-Angptl4  | Oleic acid  | $3.3 \pm 0.3 \times 10^{-6}$ | $<10^{-6}$ | $<3 \mu M$ |
|               | Palmitic acid| $2.8 \pm 0.2 \times 10^2$ | $1.7 \pm 0.2 \times 10^{-4}$ | $0.6 \mu M$ |
|               | Myristic acid| $1.4 \pm 0.2 \times 10^2$ | $2.2 \pm 0.1 \times 10^{-4}$ | $1.6 \mu M$ |
| Full-length hAngptl4 | Oleic acid | $5.7 \pm 0.1 \times 10^{-6}$ | $<10^{-6}$ | $<1.8 \mu M$ |
| BSA           | Oleic acid  | $0.8 \pm 0.2 \times 10^{-6}$ | $0.02 \pm 0.005$ | $138 \mu M$ |

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Fatty Acids Induce Conformational Changes in ccd-Angptl4 and Dissociation of Oligomers—Binding of fatty acids to hccd-Angptl4 reduced the intrinsic fluorescence of hccd-Angptl4 and caused a blue shift of the spectra. With oleic acid, the fluorescence changed immediately after mixing (supplemental Fig. 5A), although with palmitic acid the fluorescence declined for at least 10 min (supplemental Fig. 5B). The SPR experiments had demonstrated that the association rate for palmitic and oleic acid with hccd-Angptl4 was fast and comparable (Table 1). Therefore, the slower changes in the fluorescence with palmitic acid were likely due to conformational changes that occurred after formation of the hccd-Angptl4 fatty acid complex. Fig. 7A presents concentration-dependent effects of palmitic acid, myristic acid, and SDS on the fluorescence maximum wavelength of hccd-Angptl4 at equilibrium. Oleic acid caused similar fluorescence changes when its concentration was comparable with the concentration of hccd-Angptl4 (0.4 μM). However, when oleic acid was mixed with hccd-Angptl4 at higher concentrations than 0.8 μM, the solution became slightly cloudy, indicating formation of aggregates. This prevented correct fluorescence measurements. Therefore, the results obtained with oleic acid are not presented. The marked blue shift of 7–8 nm indicated that the environment around Trp-38 became more hydrophobic when hccd-Angptl4 was in complex with palmitic acid, myristic acid, or SDS. Compared with SDS and myristic acid, lower concentrations of palmitic acid caused the blue shift. This is in accordance with the affinities detected by the SPR experiments. Addition of oleic acid changed the Stern-Volmer plot to a more linear appearance and decreased the quenching constant from 6.9 to 2.2 M$^{-1}$ (Fig. 7B). This indicated a lower accessibility of acrylamide for Trp-38 in hccd-Angptl4 in complex with oleic acid, and it suggested either direct interaction between fatty acids and Trp-38 or fatty acid-induced conformational changes close to this residue.

To directly study possible conformational changes in ccd-Angptl4 induced by fatty acids, we used circular dichroism (CD) spectroscopy (Fig. 8A). Stoichiometric concentrations of oleic acid compared with ccd-Angptl4 did not affect the CD spectrum. When the molar ratio of oleic acid to ccd-Angptl4 exceeded 2, the minima at 222 and 208 nm were decreased. Analysis of the CD spectra for individual secondary structural components demonstrated that oleic acid decreased the α-helical proportion in the protein from 65 to 47%.

A lower structural stability of hccd-Angptl4 when in complex with fatty acids was also observed in experiments with limited proteolysis. The presence of stoichiometric amounts of myristic acid rendered hccd-Angptl4 more prone to cleavage by trypsin than in the absence of fatty acids (Fig. 8B). Similar results were obtained with oleic acid and palmitic acid (data not shown).

As mentioned above, the size exclusion chromatography indicated that hccd-Angptl4 eluted corresponding to the size of a 120–130-kDa protein, suggesting that it formed hexamers or even higher oligomers (Fig. 8C). In the presence of myristic acid, the monomeric peak of hccd-Angptl4 was significantly increased (Fig. 8C), demonstrating dissociation of the noncovalent hccd-Angptl4 oligomers to monomers.

**DISCUSSION**

It is well known that fatty acids up-regulate Angptl4 gene expression via interaction with peroxisome proliferator-activated nuclear receptors (48, 49). The observations in this study suggest that fatty acids may directly affect the ability of the Angptl4 protein to modulate the activity of LPL. We demonstrate, by several methods, binding of fatty acids to Angptl4. Already low concentrations (below the critical micellar concen-
FIGURE 6. **ITC analysis of the fatty acid/ccd-Angptl4 interaction.** The upper panel shows raw data of heat production during sequential 10-μl injections of hccd-Angptl4 (85 μM) into the sample cell (1.0 ml) containing 10 μM oleic acid (A) and into 10 μM myristic acid (B). C and D are respective differential heat production curves obtained after integration of the peak areas and subtraction of dilution effect of hccd-Angptl4. E and F represent cumulative heat production (ΣQ) after each addition of hccd-Angptl4. The experiments were conducted at 25 °C in 20 mM Hepes, pH 7.4, in the presence of 1.5 μM BSA.
interaction) of the fatty acids protected LPL from inactivation by Angptl4. Previous studies had shown that fatty acids at micromolar concentrations can bind to LPL and stabilize the active structure of the enzyme (39, 50). In addition, fatty acids were shown to lower the affinity of LPL for heparin/heparin sulfate and dissociate the enzyme from cell surfaces (51). It was therefore possible that the effects of fatty acids on the LPL system were due to binding of fatty acids to LPL. We show by SPR, ITC, and fluorescence measurements that the affinity of ccd-Angptl4 for fatty acids is higher than that previously reported for LPL, and even higher than that for BSA. The ccd-Angptl4/fatty acid interaction appeared to be specific in the sense that saturated fatty acids like palmitic acid and myristic acid and monounsaturated oleic acid bound much more tightly to ccd-Angptl4 than polyunsaturated fatty acids. The affinity of the different fatty acids for ccd-Angptl4 correlated well with their ability to protect LPL from inhibition by Angptl4. It is therefore likely that the protection observed on incubation with pNPB or DGGR at submicellar concentrations of fatty acids was due mainly to binding of fatty acids to Angptl4. This was supported by fluorescence measurements, demonstrating that fatty acids hinder the accessibility of the quencher acrylamide to the single Trp residue of ccd-Angptl4. This residue (Trp-38) is located close to the putative binding site for LPL (24, 25).

It has been previously shown that ccd-Angptl4 induces inactivation of LPL by causing dissociation of dimeric LPL into inactive monomers (10). Increased inactivation of LPL by ccd-Angptl4 was also detected when LPL and ccd-Angptl4 were incubated together with ester substrate DGGR (22). Here, we demonstrate that, under conditions used in our studies, ccd-Angptl4 acts mainly as an inhibitor of LPL activity when present in assay system with DGGR or pNPB as substrates. Our data show that submicellar concentrations of fatty acids could partly block the inhibiting effect of ccd-Angptl4, suggesting that binding of the ccd-Angptl4/fatty acid complexes to LPL occurred with lowered affinity. This was directly demonstrated by the SPR experiments that showed that submicellar concentrations of fatty acids reduced the rate of the formation of LPL:Angptl4 complexes, but it did not completely block the interaction of the two proteins. When fatty acid concentrations exceeded the critical micellar concentration, almost complete protection of LPL was obtained, also against the irreversible inactivation by ccd-Angptl4. This correlates well with binding of fatty acids also to LPL, which was found to occur with lower affinity than that to Angptl4. We did most of our experiments with human ccd-Angptl4 produced in bacteria, but our studies demonstrated that fatty acids bound in a similar way to mouse ccd-Angptl4 produced in eucaryotic cells (HEK293) and to full-length variants of human Angptl4 produced in yeast or mouse myeloma cells. All four proteins had a similar ability to inhibit and inactivate LPL. Thus, the slight differences in the secondary structures, detected by CD, did not influence the ability of the different variants of Angptl4 to interact with fatty acids and/or with LPL. Valuable information on the structural properties of the recombinant ccd-Angptl4s was obtained by the fluorescence measurements. Because the proteins contain only one tryptophan residue at position 38, the intrinsic fluorescence is attributed to environmental and structural properties close to this residue. The fluorescence spectral analysis together with the fluorescence quenching by acrylamide showed that the Trp-38 residues of the ccd-Angptl4s were located in environments of similar properties, probably buried inside the folded/oligomerized proteins.

Previous knowledge on the structure of Angptl4 is based on modeling, electrophoresis, and size exclusion chromatography. We present the first experimental evidence that the secondary structure of ccd-Angptl4 is mostly based on α-helices. Interestingly, the CD data predicted a coiled-coil structure for hccd-

FIGURE 7. Interaction of fatty acids with ccd-Angptl4, as measured by fluorescence. A, effect of palmitic acid, myristic acid, and SDS on the fluorescence emission maximum of hccd-Angptl4. 0.5 μM hccd-Angptl4 was mixed with various concentrations of palmitic acid, myristic acid, or SDS in 20 mM phosphate buffer, 0.15 M NaCl, pH 7.4, at 25 °C. After 20 min, the tryptophan fluorescence spectra were recorded upon excitation at 280 nm. F0 and F are fluorescence intensities determined before and after the addition of acrylamide to 0.5 μM hccd-Angptl4 in the presence or absence of 0.4 μM oleic acid. The experiments were performed at 25 °C in 20 mM Hepes, pH 7.4, containing 0.15 M NaCl. Initial fluorescence intensity ratios to residual fluorescence (F0/F) are represented as filled circles for hccd-Angptl4 alone and open circles for hccd-Angptl4 with 0.4 μM oleic acid.
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Angptl4 but not for mccd-Angptl4. Because the polypeptide chains of mccd-Angptl4 are cross-linked via disulfide bonds, it can be speculated that disulfide bonds hinder supercoiling of the N-terminal domains, at least of the recombinant protein. Both variants of the ccd-Angptl4 proteins had similar effects on LPL, indicating that neither supercoiling nor disulfide bonds are necessary for the inhibitory function.

Studies of the properties of ccd-Angptl4 by fluorescence spectroscopy, CD, gel permeability chromatography, and limited proteolysis demonstrated that fatty acids induce dramatic structural changes in the protein. The structure of the N-terminal domain of ccd-Angptl4 appeared to be rather unstable. Several previous studies report that lipid-like compounds unfold proteins of different kinds to less structured states (52, 53), probably because hydrophobic interaction is often central for protein packing.

Our SPR measurements demonstrated that the interaction between Angptl4 and fatty acids is tight and characterized by fast association and very slow dissociation, leading to formation of very stable complexes. This was clearly different from the albumin/fatty acid interaction, which was characterized by fast association and fast dissociation, resulting in rapid exchange of fatty acids between the protein and solution. The rapid exchange mechanism of the albumin/fatty acid interaction has previously been demonstrated by stopped-flow measurements (54).

The high negative enthalpy value detected for oleic acid suggests that the interaction is not entirely hydrophobic but suggested an essential contribution of ionic interaction or hydrogen bonds. Albumin has higher affinity than LPL for fatty acids. When present in excess, albumin promotes the lipolysis reaction by removing free fatty acid from the lipolysis sites (39). Thus, the main cause for the strong interference of fatty acids with the ability of ccd-Angptl4 to inhibit or inactivate LPL was most likely due to binding of fatty acids to Angptl4. Binding of oleic acid to the ccd-Angptl4 and to full-length Angptl4 had similar characteristics, indicating that fatty acid binding was mostly to the N-terminal part of Angptl4.

In biological fluids, most long-chain fatty acids are bound to albumin or cell components, but 5–10 nm is present in unbound form (55). This concentration can vary depending on the feeding/fasting status and possible pathological conditions (56, 57). Unbound fatty acids generated by lipolysis of triglycerides by LPL at the capillary endothelium may temporarily reach high values if the capacity of the adjacent tissues to assimilate the fatty acids is exceeded (58, 59). Fatty acids produced by lipolysis do not mix entirely with the plasma fatty acid pool, but a major fraction is normally taken up locally (58, 60). Because the fatty acid effect on the LPL/Angptl4 interaction is more pronounced at higher fatty acid concentrations, it is likely that fatty acids play a more important role under pathophysiological conditions than under normal conditions. The well known lack of association between plasma levels of Angptl4 and plasma triglyceride levels (35, 36) indicates that Angptl4 may control LPL activity in the subendothelial compartment where the concentration of lipoproteins is lower than in plasma. Lipolysis of triglyceride-rich lipoproteins by LPL at the capillary endothelium increases the subendothelial fatty acid concentration to higher values than in blood circulation. Our experiments performed with lipoprotein-free plasma showed that even a modest increase of the fatty acid concentration decreased the inactivation of LPL by Angptl4.
It is possible that fatty acids are involved in the nutritional regulation of LPL in adipose tissue. The activity of LPL is rapidly up-regulated in adipose tissue after a meal (27, 28). This is accompanied by an increase of the proportion of active LPL dimers to inactive monomers without major changes in the total LPL protein mass (27, 28). Postprandial endothelial lipolysis of chylomicrons by LPL results in increased flux of fatty acids into the subendothelial space. The increased flux of fatty acids could reduce the action of Angptl4 on LPL at several levels as follows: when the enzyme is bound at the luminal side of the endothelium and when the enzyme is transported through the endothelial cells or through the subendothelial space. Furthermore, the expression of ANGPTL4 is reduced in the postprandial state (10).

The oligomer state of Angptl4 in blood is not entirely known, and the picture is further complicated by the fact that Angptl4 is present both in full-length form and as fragments produced by cleavage by proprotein convertases (17, 23). Based on our data, it would be expected that fatty acids modulate the aggregation behavior of Angptl4 and thereby the conformation of the protein. This could influence on the sensitivity of Angptl4 to proteolytic enzymes. Other substances in plasma, for example lipoproteins (38), may interact with Angptl4, and this could prevent its interaction with fatty acids. An intriguing possibility is that interaction of Angptl4 with fatty acids, lipoproteins, or lipids in membranes affects the oligomerization state of the protein. Data from several groups indicate that Angptl4 oligomers linked via disulfide bonds are needed for the functions of Angptl4, e.g. for inactivation of LPL (22), and that oligomers are formed late in the secretory pathway of Angptl4, probably on cell surfaces.3

Angptl4 is a protein with many suggested functions (6). This study suggests that long-chain fatty acids have an essential role in modulation of the activity and structure of Angptl4. We demonstrate that fatty acids interfere with the interaction between LPL and Angptl4. Further studies are needed to confirm that this has physiological implications.

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REFERENCES

1. Labreuche, J., Deplanque, D., Touboul, P. J., Bruckert, E., and Amarenco, P. (2010) Association between change in plasma triglyceride levels and risk of stroke and carotid atherosclerosis. Systematic review and meta-regression analysis. *Atherosclerosis* 212, 9–15

2. DeCaterina, R., Scarano, M., Marfisi, R., Lucisano, G., Palma, F., Tatschioni, A., and Marchioli, R. (2010) Cholesterol-lowering interventions and stroke. Insights from a meta-analysis of randomized controlled trials. *J. Am. Coll. Cardiol.* 55, 198–211

3. Fruchart, J.-C., Nieren, M. C., Stroes, E. S. G., Kastelein, J. J., and Duriez, P. (2004) New risk factors for atherosclerosis and patient risk assessment. *Circulation* 109, III15–19

4. Kannell, W. B., and Vasan, R. S. (2009) Triglycerides as vascular risk factors. New epidemiologic insights. *Curr. Opin. Cardiol.* 24, 345–350

5. Yoshida, K., Shimizuogawa, T., Ono, M., and Furukawa, H. (2002) Angiopoietin-like protein 4 is a potent hyperlipidemia-inducing factor in mice and inhibitor of lipoprotein lipase. *J. Lipid Res.* 43, 1770–1772

6. Lichtenstein, L., and Kersten, S. (2010) Modulation of plasma TG lipolysis by angiopoietin-like proteins and GPIHBP1. *Biochim. Biophys. Acta* 1801, 415–420

7. Lichtenstein, L., Mattijisen, F., de Wit, N. J., Georgiadis, A., Hooiveld, G. J., van der Meer, R., He, Y., Qi, L., Köster, A., Tamsma, J. T., Tan, N. S., Müller, M., and Kersten, S. (2010) Angptl4 protects against severe proinflammatory effects of saturated fat by inhibiting fatty acid uptake into mesenteric lymph node macrophages. *Cell Metab.* 12, 580–592

8. Wang, H., and Eckel, R. H. (2009) Lipoprotein lipase. From gene to obesity. *Am. J. Physiol. Endocrinol. Metab.* 297, E271–E288

9. Davies, B. S., Beigneux, A. P., Fong, L. G., and Young, S. G. (2012) New wrinkles in lipoprotein lipase biology. *Curr. Opin. Lipidol.* 23, 35–42

10. Sukonina, V., Lookene, A., Olivecrona, T., and Olivecrona, G. (2006) Angiopoietin-like protein 4 converts lipoprotein lipase to inactive monomers and modulates lipase activity in adipose tissue. *Proc. Natl. Acad. Sci.* 103, 17450–17455

11. Chu, A., Lam, M. C., Chan, K. W., Wang, Y., Zhang, J., Hoo, R. L., Xu, J. Y., Chen, B., Chow, W. S., Tso, A. W., and Lam, K. S. (2005) Angiopoietin-like protein 4 decreases blood glucose and improves glucose tolerance but induces hyperlipidemia and hepatic steatosis in mice. *Proc. Natl. Acad. Sci. U.S.A.* 102, 6086–6091

12. Gealekman, O., Burkart, A., Chounard, M., Nicoloro, S. M., Straubhaar, J., and Corvera, S. (2008) Enhanced angiogenesis in obesity and in response to PPAR activators through adipocyte VEGF and ANGPTL4 production. *Am. J. Physiol. Endocrinol. Metab.* 295, E1056–E1064

13. Chomel, C., Cazes, A., Faye, C., Bignon, M., Gomez, E., Ardidie-Robouant, C., Barret, A., Ricard-Blum, S., Muller, L., Germain, S., and Monnot, C. (2009) Interaction of the coiled-coil domain with glycosaminoglycans protects angiopoietin-like 4 from proteolysis and regulates its antiangiogenic activity. *FASEB J.* 23, 940–949

14. Nakayama, T., Hirakawa, H., Shibata, K., Nazneen, A., Abe, K., Nagayasu, T., and Taguchi, T. (2011) Expression of angiopoietin-like 4 (ANGPTL4) in human colorectal cancer. ANGPTL4 promotes venous invasion and distant metastasis. *Oncol. Rep.* 25, 929–935

15. Goh, Y. Y., Pal, M., Chong, H. C., Zhu, P., Tan, M. J., Punuguri, L., Tan, C. K., Huang, R. L., Sze, S. K., Tang, M. B., Ding, J. L., Kersten, S., and Tan, N. S. (2010) Angiopoietin-like 4 interacts with matrix proteins to modulate wound healing. *J. Biol. Chem.* 285, 32999–33009

16. Yoon, J. C., Chickering, T. W., Rosen, E. D., Dussault, B., Qin, Y., Soukas, A., Friedman, J. M., Holmes, W. E., and Spiegelman, B. M. (2000) Peroxisome proliferator-activated receptor γ target gene encoding a novel angiopoietin-related protein associated with adipose differentiation. *Mol. Cell. Biol.* 14, 5343–5349

17. Ge, H., Yang, G., Huang, L., Motola, D. L., Pourbahrami, T., and Li, C. (2004) Oligomerization and regulated proteolytic processing of angiopoietin-like protein 4. *J. Biol. Chem.* 279, 2038–2045

18. Mandard, S., Zandbergen, F., Tan, N. S., Escher, P., Patsouris, D., Koenig, W., Kleemann, R., Bakker, A., Veenman, F., Wahl, H., Müller, M., and Kersten, S. (2004) The direct peroxisome proliferator-activated receptor target fasting-induced adipose factor (FIAF/PARG/ANGPTL4) is present in blood plasma as a truncated protein that is increased by fenofibrate treatment. *J. Biol. Chem.* 279, 34411–34420

19. Ge, H., Cha, J. Y., Gopal, H., Harp, C., Yu, X., Repa, J. J., and Li, C. (2005) Differential regulation and properties of angiopoietin-like proteins 3 and 4. *J. Lipid Res.* 46, 1484–1490

20. Ge, H., Yang, G., Yu, X., Pourbahrami, T., and Li, C. (2004) Oligomerization-state dependent hyperlipidemic effect of angiopoietin-like protein 4. *J. Biol. Chem.* 279, 13213–13222

21. Shan, L., Yu, X. C., Liu, Z., Hu, Y., Sturgis, L. T., Miranda, M. L., and Liu, Q. (2009) The angiopoietin-like proteins ANGPTL3 and ANGPTL4 inhibit lipoprotein lipase activity through distinct mechanisms. *J. Biol. Chem.* 284, 1419–1424
Interaction of Fatty Acids with Angiopoietin-like Protein 4

23. Lei, X., Shi, F., Basu, D., Huq, A., Routhier, S., Day, R., and Jin, W. (2011) Proteolytic processing of angiopoietin-like protein 4 by proprotein convertases modulates its inhibitory effects on lipoprotein lipase activity. J. Biol. Chem. 286, 15747–15756

24. Lee, E. C., Desai, U., Golobov, G., Hong, S., Feng, X., Yu, X. C., Gay, I., Wilganowski, N., Gao, C., Du, L. L., Chen, J., Hu, Y., Zhao, S., Kirkpatrick, L., Schneider, M., Zambrówicz, B. P., Landes, G., Powell, D. R., and Sonnenburg, W. K. (2009) Identification of a new functional domain in angiopoietin-like 3 (ANGPTL3) and angiopoietin-like 4 (ANGPTL4) involved in binding and inhibition of lipoprotein lipase (LPL). J. Biol. Chem. 284, 13735–13745

25. Yao, M. H., Wang, Y., Lam, K. S., Zhang, J., Wu, D., and Xu, A. (2009) A highly conserved motif within the N2-terminal coiled-coil domain of angiopoietin-like protein 4 confers its inhibitory effects on lipoprotein lipase by disrupting the enzyme dimerization. J. Biol. Chem. 284, 11942–11952

26. Romero, S., Pennacchio, L. A., Fu, Y., Boerwinkle, E., Tybjaerg-Hansen, A., Hobs, H. H., and Cohen, J. C. (2007) Population-based resequencing of ANGPTL4 uncovers variations that reduce triglycerides and increase HDL. Nat. Genet. 39, 513–516

27. Bergö, M., Olivecrona, G., and Olsvigcrona, T. (1996) Forms of lipoprotein lipase in rat tissues. In adipose tissue the proportion of inactive lipase increases on fasting. Biochim. Biophys. Acta 1313, 893–898

28. Bergö, M., Wu, G., Ruge, T., and Olsvigcrona, T. (2002) Down-regulation of adipose tissue lipoprotein lipase during fasting requires that a gene, separate from the lipase gene, is switched on. J. Biol. Chem. 277, 11927–11932

29. Dutton, S., and Trayhurn, P. (2008) Regulation of angiopoietin-like protein 4 fasting-induced adipose factor (ANGPTL4/FIAF) expression in mouse adipose tissue and 3T3-L1 adipocytes. Br. J. Nutr. 100, 18–26

30. Kersten, S., Desvergne, B., and Muñoz, M., van, Hendriks, H. F., Miyazaki, R., Goto, Y., and Naiki, H. (2009) Caloric restriction and exercise increase plasma ANGPTL4 levels in humans via elevated fatty acids. Arterioscler. Thromb. Vasc. Biol. 29, 969–974

31. Sober, G., von, Christenson, R. H., Mockel, M., Danne, O., and Jaffe, A. S. (2005) Future biomarkers for detection of ischemia and risk stratification in acute coronary syndrome. Clin. Chem. 51, 810–824

32. Teusink, B., Voshol, P. J., Dahlmans, V. E., Rensen, P. C., Pijl, H., Romijn, J. A., and Havelvees, I. M. (2003) Contribution of fatty acids released from lipolysis of plasma triglycerides to total plasma fatty acid flux and tissue-specific fatty acid uptake. Diabetes 52, 614–620

Abstracts of the XV International Symposium on Atherosclerosis, Boston, MA, June 14–18, 2009, p. e238

33. Bengtsson, G., and Olsvigcrona, T. (1980) Lipoprotein lipase. Mechanism of product inhibition. Eur. J. Biochem. 106, 557–562

34. Bengtsson-Olsvigcrona, G., and Olsvigcrona, T. (1991) Phospholipase activity of milk lipoprotein lipase. Methods Enzymol. 197, 345–356

35. Gill, S. C., and von, Hoppel, P. H. (1989) Calculation of protein extinction coefficients from amino acid sequence data. Anal. Biochem. 182, 319–326

36. Goldstein, J. L., Basu, S. K., and Brown, M. S. (1983) Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. Methods Enzymol. 98, 241–260

37. Lakowicz, J. R. (2006) Principles of Fluorescence Spectroscopy, pp. 278–292, Springer-Verlag, Inc, New York

38. van, der Vusse, G. J. (2009) Albumin as fatty acid transporter. Drug Metab. Pharmacokinet. 24, 300–307

39. Edwards, K., Chan, R. Y., and Sawyer, W. H. (1994) Interactions between fatty acids and lipoprotein lipase. Specific binding and complex formation. Biochemistry 33, 13304–13311

40. Bojesen, E., and Bojesen, I. N. (2006) Albumin binding of long-chain fatty acids. Thermodynamics and kinetics. J. Phys. Chem. 100, 17981–17985

41. Aki, H., and Yamamoto, M. (1994) Thermodynamic characterization of drug binding to human serum albumin by isothermal titration microcalorimetry. J. Pharm. Sci. 83, 1712–1716

42. Kersten, S., Lichtenstein, L., Steenbergen, E., Madde, K., Hendriks, H. F., Hesselink, M. K., Schrauwenn, P., and Müller, S. (2009) Caloric restriction and exercise increase plasma ANGPTL4 levels in humans via elevated fatty acids. Arterioscler. Thromb. Vasc. Biol. 29, 969–974

43. Anderson, L. M., Degenhardt, T., Koppenn, A., Kalkhoven, E., Desvergne, B., Müller, M., and Kersten, S. (2009) Peroxisome proliferator-activated receptor β/δ (PPARβ/δ) but not PPARα serves as a plasma free fatty acid sensor. Mol. Cell. Biol. 29, 6257–6267

44. Shimada, K., Lanzillo, J. I., Douglas, W. H., and Fanburg, B. L. (1982) Stabilization of lipoprotein lipase by endothelial cells. Biochem. Biophys. Acta 710, 117–121

45. Saxena, U., Witte, L. D., and Goldberg, I. J. (1989) Release of endothelial cell lipoprotein lipase by plasma lipoproteins and free fatty acids. J. Biol. Chem. 264, 4349–4355

46. Kim, Y. J., and Takahashi, R. (2006) Role of polysaturated fatty acids for misfolded protein aggregations. Implication for neurodegenerative diseases. Ann. N.Y. Acad. Sci. 1086, 11–20

47. Hasegawa, K., Tsutsumi-Yasuhara, S., Oohoshi, T., Ohhashi, Y., Kimura, H., Takahashi, N., Yoshida, H., Miyazaki, R., Goto, Y., and Naiki, H. (2008) Growth of β3-microglobulin-related amyloid fibrils by nonesterified fatty acids at a neutral pH. Biochem. J. 416, 307–315

48. Demant, E. J., Richieri, G. V., and Kleinfield, A. M. (2002)Stopped-flow kinetic analysis of long-chain fatty acid dissociation from bovine serum albumin. Biochem. J. 363, 809–815

49. Richieri, G. V., and Kleinfield, A. M. (1995) Unbound free fatty acid levels in human serum. J. Lipid Res. 36, 229–240

50. Kleinfield, A. M., Protho, D., Brown, D. L., Davis, R. C., Richieri, G. V., and DeMaria, A. (1996) Increases in serum unbound free fatty acid levels following coronary angioplasty. Am. J. Cardiol. 78, 1235–12354

51. Apple, F. S., Wu, A. H., Mair, J., Ravkilde, J., Panteghini, M., Tate, J., Pagani, F., Christenson, R. H., and Jaffe, A. S. (1998) Future biomarkers for detection of ischemia and risk stratification in acute coronary syndrome. Clin. Chem. 51, 810–824

52. Teusink, B., Voshol, P. J., Dahlmans, V. E., Rensen, P. C., Pijl, H., Romijn, J. A., and Havelvees, I. M. (2003) Contribution of fatty acids released from lipolysis of plasma triglycerides to total plasma fatty acid flux and tissue-specific fatty acid uptake. Diabetes 52, 614–620