Original Article

MOLECULAR METABOLISM

Angiopoietin-like 4 directs uptake of dietary fat away from adipose during fasting

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ABSTRACT

Objective: Angiopoietin-like 4 (ANGPTL4) is a fasting-induced inhibitor of lipoprotein lipase (LPL) and a regulator of plasma triglyceride metabolism. Here, we examined the kinetics of *Angptl4* induction and tested the hypothesis that ANGPTL4 functions physiologically to reduce triglyceride delivery to adipose tissue during nutrient deprivation.
Methods: Gene expression, LPL activity, and triglyceride uptake were examined in fasted and fed wild-type and *Angptl4*$^{-/-}$ mice.
Results: *Angptl4* was strongly induced early in fasting, and this induction was suppressed in mice with access to food during the light cycle. Fasted *Angptl4*$^{-/-}$ mice manifested increased LPL activity and triglyceride uptake in adipose tissue compared to wild-type mice.
Conclusions: *Angptl4* is induced early in fasting to divert uptake of fatty acids and triglycerides away from adipose tissues.

Keywords Plasma triglycerides; Lipoprotein metabolism; Lipolysis; Lipase inhibition

1. INTRODUCTION

Misregulation of plasma triglyceride metabolism and fatty acid delivery has been implicated in several metabolic diseases, including metabolic syndrome, diabetes mellitus, and atherosclerosis [1,2]. The enzyme lipoprotein lipase (LPL) is positioned at the nexus of plasma triglyceride delivery, hydrolyzing plasma lipoprotein triglycerides and releasing fatty acids for uptake into heart, skeletal muscle, and adipose tissue. LPL-mediated hydrolysis normally occurs on the luminal surface of capillary endothelial cells where LPL is anchored by GPIHBP1, an endothelial cell GPI-anchored protein responsible for transporting LPL across endothelial cells [3,4].

Angiopoietin-like 4 (ANGPTL4), also known as fasting-inducible adipose factor (FIAF), is a fasting induced inhibitor of LPL and a regulator of triglyceride metabolism [5–7]. ANGPTL4 is most highly expressed in adipose tissue and liver, but it is also expressed at lower levels in muscle, heart, kidney, and intestine [5,8], and circulates in plasma [5]. Plasma triglycerides are elevated in mice overexpressing ANGPTL4, whereas *Angptl4*$^{-/-}$ mice display reduced plasma triglyceride levels [7]. ANGPTL4 inactivates LPL by accelerating the dissociation of active LPL dimers to inactive monomers [9]. ANGPTL4 expression increases markedly upon fasting leading to the hypothesis that ANGPTL4 is involved in regulating fatty acid delivery in the fasted state [5].

In humans, inactivating mutations in ANGPTL4 are associated with lower plasma triglycerides [10–12] and lower incidence of coronary artery disease [11–13]. Thus, it has been proposed that targeting ANGPTL4 activity may be a useful way to therapeutically increase LPL-driven triglyceride clearance, lower plasma triglycerides, and lower the risk of coronary disease [11,12,14,15]. However, as LPL-driven ectopic lipid deposition can potentially lead to detrimental effects, including skeletal muscle insulin resistance [16], cardiac lipotoxicity [17], and severe inflammatory responses [18], understanding where and when ANGPTL4 normally acts is essential.

In this study, we investigate the physiological mechanisms by which ANGPTL4 regulates plasma triglycerides. We analyze the tissue-specific kinetics of fasting-induced *Angptl4* gene expression, and examine the effects of ANGPTL4 deficiency on tissue-specific lipase activity and triglyceride delivery.

2. MATERIALS AND METHODS

2.1. Mice

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Iowa and were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were group housed in a controlled environment with a 12/12 light/dark cycle, with food and water provided *ad libitum* during non-fasting conditions. During fasting conditions, water was provided *ad libitum*.

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Abbreviations: ANGPTL4, angiopoietin-like 4; LPL, lipoprotein lipase; GPIHBP1, glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1; sWAT, subcutaneous white adipose tissue; gWAT, gonadal white adipose tissue; mWAT, mesenchymal white adipose tissue; BAT, brown adipose tissue

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C57BL/6J mice were obtained from Jackson Laboratories. *Gpihbp1*$^{-/-}$ mice (B6;129S5-*Gpihbp1*^{tm1Lex}/Mmucd) [19,20], and *Angptl4*$^{-/-}$ mice (B6;129S5-*Angptl4*$^{Gt(OST352973)Lex}$/Mmucd) [19,21] were generated by breeding from strains obtained from the Mutant Mouse Resource and Research Center (mmrrc.org). Age-matched wild-type littermates were used as controls for *Angptl4*$^{-/-}$ mice.

2.2. Plasma triglyceride assays

Angptl4$^{-/-}$ mice and wild-type littermates were fasted for 4 h (fasted group) or fasted for 6 h and then fed normal chow for 2 h (refed group). Blood was collected via tail-nick into EDTA-coated collection tubes. After centrifugation at 1500×*g* for 15 min at 4 °C to pellet the cells, plasma from each mouse was combined with Infinity™ Triglyceride Reagent (Thermo Scientific TR22421) according to the manufacturer's instructions. Samples were incubated at 37 °C for 10 min and absorbance was measured at 500 nm. Triglyceride concentrations were determined by comparison to a standard curve prepared from a triolein standard (Nu-Chek Prep, Lot T-235-N13-Y).

2.3. RNA isolation and qPCR analysis

Mouse tissue samples were frozen in liquid nitrogen and pulverized using a Bessman tissue pulverizer. Crushed tissue was resuspended in Trizol (Ambion, 15596-018) and processed according to the manufacturer's instructions. After assessing mRNA concentration and quality using a Nanodrop spectrophotometer (ThermoScientific), cDNA was prepared using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Part No. 4368813). qPCR was performed (Invitrogen, SYBR GreenER qPCR Supermix, 11762100) according to the manufacturer's specifications using an Applied Biosystems 7900HT Fast Real-Time PCR System (Iowa Institute of Human Genetics). Relative expression was calculated with the ΔΔct method [22] using *CycloA* as the reference gene.

2.4. Lipase activity assay

Frozen tissue samples were crushed and resuspended in LPL assay buffer (25 mM NH_4Cl, 5 mM EDTA, 0.01% SDS, 45 U/mL heparin, 0.05% 3-(N,N-Dimethylmyristylammonio) propanesulfonate zwittergent detergent (Acros Organics, 427740050)) containing Mammalian ProteaseArrest (GBiosciences, cat no. 786-331). The tissue suspension was mixed by vortexing and incubated on ice for 30 min, with intermittent disruption with surgical scissors. The resulting lysate was centrifuged at 15,000×*g* for 15 min at 4 °C to pellet cellular debris. Lipase activity assays were performed on the supernatants as previously described [23]; supernatants were combined with a working buffer composed of 0.6 M NaCl, 80 mM Tris—HCl pH 8, 6% fatty-acid free BSA and an EnzChek lipase fluorescent substrate (Molecular Probes, E33955). Fluorescence was measured over 30 min at 37 °C on a SpectraMax i3 plate reader (Molecular Devices). Relative lipase activity was calculated by subtracting background (calculated by reading fluorescence of a sample with no LPL) and then calculating the slope of the curve between the 5 and 13 min reads. The data were graphed as the average of slopes for each group.

2.5. Preparation of ^{3}H-Labeled intralipid

^{3}H-Intralipid was prepared by mixing [9,10-3H(N)]-Triolein (Perkin Elmer, NET431001MC) with 5% Intralipid (prepared fresh from Intralipid® 20% (NDC 0338-0519-03)) in a ratio of 1 μCi triolein to 10 μL of Intralipid. The mixture was sonicated briefly on low power 3 × 20 s, with 1 min on ice between each round of sonication. The mixture was centrifuged briefly and diluted 10-fold in saline to prepare a 0.5% stock.

2.6. Preparation of ^{3}H-Labeled chylomicrons

Gpihbp1$^{-/-}$ mice were fasted 4 h and then gavaged with 100 μCi of [9,10-3H(N)]-Triolein (Perkin Elmer, NET431001MC) suspended in olive oil. After 4 h, mice were anesthetized, and blood was collected by cardiac puncture. Blood was diluted 1:10 with 0.5 M EDTA (pH 8.0) and centrifuged 1500×*g* for 15 min at 4 °C to pellet blood cells. The plasma was then transferred to ultracentrifuge tubes and mixed 1:1 with PBS. After centrifugation at 424,000×*g* for 2 h at 10 °C, the chylomicrons form an upper layer. The chylomicron layer was resuspended in fresh PBS and the centrifugation was repeated. Following the second centrifugation, the chylomicron layer was resuspended in PBS to the original plasma volume. Protein content was assayed using the BioRAD DC Protein Assay (BioRAD, 5000116). Radioactivity was determined in BioSafe II scintillation fluid (RPI, 111195) on a Beckman-Coulter Liquid Scintillation Counter (BCLSC6500).

2.7. Triglyceride clearance assay

Wild-type and *Angptl4*$^{-/-}$ mice were fasted 4 h (fasted group) or were fasted for 6 h and then returned to chow for 2 h (refed group). Mice were anesthetized with isoflurane and injected retro-orbitally with 200 μL 0.5% ^{3}H-Intralipid (see Section 2.5) or 100 μL of ^{3}H-chylomicron suspension (see Section 2.6). Proparacaine hydrochloride ophthalmic solution, USP 0.5% (AKORN, 17478-263-12) was used to minimize discomfort both during and after injection. Blood samples were taken via tail-nick at 1, 5, 10, and 15 min after injection. Blood samples were assayed in BioSafe II scintillation fluid on a Beckman-Coulter Scintillation Counter. After the last blood draw, the mice were anesthetized with isoflurane, and tissues were harvested and weighed. A portion of each tissue was then weighed and placed in 2:1 chloroform:methanol overnight at 4 °C. 1 mL of 2 M $CaCl_2$ was then added to each sample to separate organic and aqueous layers. The samples were centrifuged for 10 min at 1500 rpm, and the upper aqueous layer was mixed with BioSafe II scintillation fluid and assayed on a Beckman-Coulter Scintillation Counter. The lower organic layer was evaporated overnight to remove chloroform, and the remaining sample was resuspended in scintillation fluid and assayed in BioSafe II scintillation fluid on a Beckman-Coulter Liquid Scintillation Counter. Aqueous and organic fractions were combined to obtain the total uptake CPM. CPM were measured for an aliquot representing 10% (by volume) of the chylomicrons injected into each mouse. This value was used to normalize the radiolabel data across mice.

2.8. Triglyceride uptake after gavage with ^{3}H-Triolein

Wild-type and *Angptl4*$^{-/-}$ mice were fasted 4 h, beginning at the onset of the light cycle. After 4 h, the mice were gavaged with 2 μCi of [9,10-3H(N)]-Triolein (Perkin Elmer, NET431001MC) in 100 μL olive oil. After 4 h, the mice were anesthetized with isoflurane, and tissues were harvested and weighed. Tissues were then analyzed for radiolabel as described in Section 2.7.

2.9. Statistics and outlier identification

Statistics and outlier identification were performed using Graphpad Prism. Statistical significance was tested using Student's T-test unless otherwise indicated. Outliers were identified using Grubbs test and were excluded from graphs and from statistical analysis. The number

