Differential regulation and properties of angiopoietin-like proteins 3 and 4

Hongfei Ge,[*,†] Ji-Young Cha,[*,†] Harini Gopal,[*,†] Christopher Harp,[*,†] Xinxin Yu,[*,†] Joyce J. Repa,[*,†,§] and Cai Li[1,2,*,†,§]

Touchstone Center for Diabetes Research,* Department of Physiology,† and Department of Internal Medicine,§ The University of Texas Southwestern Medical Center, Dallas, TX 75390-8854

Abstract Angiopoietin-like protein 3 and 4 (Angptl3 and Angptl4) are two members of the angiopoietin-like family of proteins. These two closely related proteins have been reported to similarly affect lipid metabolism through their capacity to inhibit lipoprotein lipase. We undertook a series of studies to compare the structure, function, and regulation of Angptl3 and Angptl4. Previously, we reported that Angptl4 exists as variable-sized oligomers that contain intermolecular disulfide bonds. We now have evidence that although there are no intermolecular disulfide bonds evident in Angptl3, higher molecular weight forms do exist. In addition, Angptl4 exhibits a widespread distribution of tissue expression, while Angptl3 is exclusively expressed in the liver. Treatments with various ligands of nuclear receptors reveal that Angptl3 is a target gene of liver X receptor, while Angptl4 expression is activated by ligands of all peroxisome proliferator-activated receptors. Expression of Angptl4 in adipose tissue and liver is induced by fasting, while Angptl3 expression is not appreciably affected by nutritional status. We suggest that the differential regulation of Angptl3 and Angptl4 by sites of expression, nutritional status, and ligands of nuclear receptors may confer unique roles of each in lipoprotein metabolism.—Ge, H., J.-Y. Cha, H. Gopal, C. Harp, X. Yu, J. J. Repa, and C. Li. **Differential regulation and properties of angiopoietin-like proteins 3 and 4. *J. Lipid Res.* 2005.** 46: **1484–1490.**

Supplementary key words Angptl3 • Angptl4 • LPL • peroxisome proliferator-activated receptor • liver X receptor • gel filtration • nutritional status

Angiopoietin-like protein 3 and 4 (Angptl3 and Angptl4) are two members of the angiopoietin-like family of proteins that inhibit lipoprotein metabolism (1–4). Angptl3 and Angptl4 share 31% amino acid sequence identity, consisting of a signal sequence at the N-terminus, followed by a coiled-coil domain, and a fibrinogen-like domain at the carboxyl terminus. Angptl3 is a target of liver X receptor (LXR) (5, 6), while expression of Angptl4 is induced by ligands of peroxisome-activated receptor (PPAR)α, PPARβ/δ, and PPARγ under different settings as well as by nutritional status (7–10).

Two independent studies have shown that Angptl3 is a transcriptional target of LXR, a member of the nuclear receptor superfamily activated by oxysterols. Both cholesterol feeding, which generates ligands for LXR, and treatment of mice with T0901317, a synthetic ligand of LXR, induced hepatic Angptl3 expression as well as a rise of plasma triglyceride (TG) levels (6). Promoter analysis on both the mouse and the human ANGPTL3 genes revealed the presence of a single LXR binding site that is required for LXR responsive element–mediated regulation (5, 6). Treatment of Angptl3 knockout mice with T0901317 failed to elicit the hypertriglyceridemia seen in wild-type mice, demonstrating that Angptl3 is responsible for the hyperlipidemic effects of LXR ligands (5). In mouse models of insulin deficiency, such as streptozotocin-induced diabetes, or models of insulin resistance, such as *db/db* mice, Angptl3 mRNA levels are increased by 2- to 3-fold, with a similar increase of Angptl3 protein levels in the liver (11). Treatment of *ob/ob* mice or rat hepatoma cells with leptin reduces Angptl3 expression, as does insulin on rat hepatoma cells (12).

Angptl4 was identified independently during a search of additional angiopoietin-related protein from embryonic cDNAs (13), as a gene that is induced by fasting (7), and during 3T3-L1 preadipocyte differentiation (8), and was thus named HFARP (hepatic fibrinogen/angiopoietin-related protein), FIAF (fasting-induced adipose factor), and PGAR (PPARγ-angiopoietin related) (7, 8, 13). Angptl4 is expressed predominantly in adipose tissue, liver, and placenta, and is repressed by leptin (8). Expres-

Manuscript received 6 January 2005 and in revised form 23 March 2005 and in re-revised form 19 April 2005.

Published, JLR Papers in Press, May 1, 2005.
DOI 10.1194/jlr.M500005-JLR200

[1]Present address of Cai Li: Departments of Metabolic Disorders, Merck Research Laboratories, RY80-A19, P.O. Box 2000, 126 E. Lincoln Avenue, Rahway, NJ 07065.
[2]To whom correspondence should be addressed.
e-mail: cai_li@merck.com

sion of Angptl4 has also been reported in keratinocytes, intestine, and pituitary gland (10, 14, 15). Suggestive of a regulatory role in new blood vessel formation, Angptl4 expression is increased by about 20-fold in human fetal cardiac cells and neonatal rat cardiomyocytes when cultured in a hypoxia chamber (1% O_2 vs. 21% O_2, which is normoxic) (16). While three transcriptional start sites have been identified in murine Angptl4 gene (17), a *cis*-regulatory element controlling Angptl4 expression was found within intron 3, which binds to both PPARα and PPARγ via gel shift analysis as well as chromatin immunoprecipitation assay (18). These observations raised the possibility that Angptl4 may play a role in fuel partitioning, the body's responses to agonists of nuclear receptors, and angiogenesis.

A physiological role of Angptl4 in fuel partitioning is also supported by the observation that germ-free mice exhibit increased expression of Angptl4 in the intestine and a 40% decrease of body fat. However, Angptl4 knockout mice raised under the same conditions have the same percentage of body fat as wild-type mice. These results suggest that expression of Angptl4 in the intestine may interfere with fat storage, and that Angptl4 may also play a role in energy acquisition between microbiota and host interactions (14). More direct evidence of a role for Angptl4 in fuel partitioning comes from our transgenic study in which cardiac-directed Angptl4 overexpression inhibits lipoprotein-derived FFA delivery to the heart (19).

To further understand the molecular mechanisms underlying the different biological activities of Angptl3 and Angptl4, we determined the expression patterns of both proteins under different nutritional states, the capacity of each to assemble into higher order structures, and the differential regulation by ligands of nuclear hormone receptors.

MATERIALS AND METHODS

Reagents

PCR primers were synthesized by IDT Technologies (Coralville, IA). Restriction endonucleases were from New England Biolabs, Inc. (Beverly, MA). Taq DNA polymerase, DNase I, protease inhibitor cocktail, and c-*myc*-HRP antibody were from Roche (Indianapolis, IN). Trizol reagent and Superscript II RNase H^- reverse transcriptase were from Invitrogen (Carlsbad, CA). FLAG-HRP antibody was from Sigma (St. Louis, MO). ECL-Western blotting reagents, Probe Quant G-50 column, Hybond-N+ nylon membrane, and Superdex 200 10/300 GL column were from Amersham Biosciences (Piscataway, NJ). SYBR Green PCR Master Mix was from Applied Biosystems (Foster City, CA). Nitrocellulose membrane was from Schleicher and Schuell (Keene, NH). Blue Bio film for ECL detection was from Denville Scientific Inc. (Metuchen, NJ). [α-^{32}P] dCTP (3000 Ci/mM, 20 mCi/ml) was from MP Biomedicals (Irvine, CA). Intralipid (20% iv fat emulsion) was from Baxter (Deerfield, IL). FFA assay kit was from Wako (Richmond, VA). Nuclear hormone receptor ligands were obtained from the following sources: T1317 was from Caymen Chemical (Ann Arbor, MI), GW0742 was from Dr. Steven Kliewer (UT Southwestern Medical Center, Dallas, TX) and/or Timothy Willson (GlaxoSmithKline, Research Triangle Park, NC), and pioglitazone was from Takeda Pharmaceuticals (Lincolnshire, IL).

Construction of adenoviral expression vectors

Adenoviruses encoding full-length rat Angptl4 and mouse Angptl3 with both FLAG and *c-myc* tags (Ad-FLAG-Angptl4-myc and Ad-FLAG-Angptl3-myc) were constructed as described (20). Full-length mouse Angptl3 cDNA was amplified from mouse liver cDNA using primers CL435 (5′GGAATTCATCCAGAGTGGATCCAGACCTT-3′) and CL437 (5′-GCTCTAGAGGTGGTGGGCTGGAGCATCAT-3′).

Expression and detection of Angptl3 and Angptl4 proteins in HEK293 cells

HEK293 cells were cultured in 6-well plates until 80–90% confluent. Culture medium was replaced with fresh Dulbecco's modified Eagle medium with or without 5% FBS before adenoviruses encoding Angptl3 or Angptl4 were added at a multiplicity of infection of 100. In some cases, protease inhibitor cocktail was added following the manufacturer's instructions. Forty-eight hours after virus infection, conditioned medium was collected and centrifuged to obtain supernatant. Samples were incubated with SDS loading buffer either with (reduced) or without (nonreduced) β-mercaptoethanol and subjected to 12% SDS-PAGE. After electrophoresis, samples were transferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in TBST, and Angptl3 or Angptl4 protein was detected with a *c-myc*-HRP or FLAG-HRP antibody using an ECL detection system.

Size exclusion chromatography

A superdex 200 10/300 GL column was equilibrated with 50 mM Na-phosphate (pH 7.0) containing 0.15 M NaCl at 4°C at a flow rate of 0.5 ml/min. The applied sample volumes were 200 μl. Fractions of 300 μl were collected, from which 20 μl per fraction was used for Western blotting. The column was calibrated by measuring the absorbance of commercially available protein standards.

Northern blotting

Twenty μg (liver) or 5 μg (adipose tissue) of total RNA was loaded onto a 1% formaldehyde agarose gel and transferred to Hybond-N+ membrane. cDNA fragments specific to Angptl3 and Angptl4 were generated via RT-PCR using mouse liver RNA as template. Probes were synthesized by PCR and labeled with random primer labeling kit using [α-^{32}P] dCTP (3000 Ci/mM, 20 mCi/ml). Labeled probe was purified from free radionucleotides with a Probe Quant G-50 column. Filters were prehybridized and hybridized in RapidHyb buffer in a 65°C oven. Washed filters were exposed to X-ray film at −70°C.

Animal treatment procedure

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center at Dallas. C57BL/6 mice (Jackson Laboratory) between 9 and 11 weeks of age were housed and fed standard rodent chow for at least 1 week before study initiation. Fasted animals were deprived of food for 24 h, starting at the beginning of the dark cycle. The refed group was fasted for 24 h and then allowed to feed ad libitum for another 6 h before being killed. For gavage experiments, each animal group (n = 4) was dosed orally (200 μl/20 g) with different agonist compounds in 1% methylcellulose: PPARα agonist fenofibrate at 200 mg/kg body weight, PPARγ agonist pioglitazone at 20 mg/kg body weight, PPARδ agonist GW0742 at 5 mg/kg body weight, and LXR agonist T1317 at 50 mg/kg body weight. Ligands are pro-

vided once daily at the beginning of the dark cycle for 3 days. On the fourth day, mice received a fourth dose by oral gavage at the onset of light cycle and sacrificed 3 h later to collect tissues.

Quantitative real-time PCR

Quantitative real-time PCR was performed as previously described (21). Briefly, total RNA was isolated from cultured cells or mouse tissues, treated with DNase I, and reverse-transcribed using Superscript II RNase H$^-$ reverse transcriptase. Quantitative real-time PCR was performed using an ABI Prism 7000 Detection System (Applied Biosystems). Each reaction was performed in a volume of 20 μl that contained 50 ng cDNA, 10 μl SYBR green PCR Master Mix, and 150 nM of each PCR primer. Levels of various mRNAs were normalized to those of cyclophilin (mouse) or 36B4 (rat). Primers sequences are as follows: 5′-AGCACACAGACCTGATGTTTTCTAC-3′ and 5′-CCACCTGAGTAACTTTCTGGACAGT-3′ for mouse Angptl3; 5′-GGACTGGGATGGCAATGC-3′ and 5′-CCTCACCCCCCAAATGG-3′ for mouse Angptl4; 5′-GGAGATGGCACAGGAGGAA-3′ and 5′-GCCCGTAGTGCTTCAGCTT-3′ for mouse cyclophilin; 5′-TGGCGCTCCAACACAGAGT-3′ and 5′-CAGGCGGGTTCTGAACCA-3′ for rat Angptl3; 5′-TGGGTGCCACCAATGTTTC-3′ and 5′-TGGTCTTGGTCCCAGGTAGAGA-3′ for rat Angptl4; and 5′-CACCTTCCCACTGGCTGAA-3′ and 5′-TCCTCCGACTCTTCCTTTGC-3′ for rat 36B4. Calculations were performed via the comparative CT method (User Bulletin #2, Perkin Elmer).

Assay for LPL-derived FFA from artificial triglyceride substrate

Control C2C12 cells or C2C12 cells stably expressing LPL were obtained from Dr. R. Eckel (22). Cells were seeded in 24-well plates and allowed to grow to about 80% confluence. Adenoviruses encoding GFP control, Angptl3, or Angptl4 were added to cells at a multiplicity of infection of 1,000. Forty-eight h after infection, the medium was replaced by 200 μl DMEM containing 200 mg/dl triglyceride (Intralipid), 12 mM glucose, 5% fasted rat serum (to provide apolipoprotein C-II) and 5 U/ml heparin. Cells were maintained in experimental media at 37°C in an atmosphere of 5% CO_2 and 100% humidity Samples were taken from the media at 0, 2, 4, 6, and 8 h. Media samples were immediately frozen and stored at −20°C until analyzed. Media FFA was measured via enzymatic methods using a commercial kit from Wako.

RESULTS

Angptl3 and Angptl4 share structural similarity but different expression and regulation

A total of nine members of the angiopoietin and angiopoietin-like family of proteins have been identified, designated angiopoietins 1 to 3 (Agpt1 to Agpt3) and angiopoietin-like proteins 1 through 6 (Angptl1 to Angptl6). Angptl5, a heart-specific form, has been identified only in humans. We performed phylogenetic analysis of all nine members of this family of proteins (**Fig. 1**). The founding members, agpt1 and -2, which bind Tie 2 and play critical roles in vascular genesis (23), are closely related. Angptl3 and Angptl4, both shown to cause a rise of plasma triglyceride levels (3, 4), are also most closely related. The physiological activities of other members are not known.

We determined the expression profile of Angptl3 and Angptl4 in liver and fat under different nutritional conditions. While Angptl4 is expressed both in liver and adipose tissue, expression of Angptl3 can be detected only in liver via Northern blot analysis (**Fig. 2**). Furthermore, Angptl4 expression is induced by fasting in both adipose tissue and liver, while that for Angptl3 is not altered by nutritional status (Fig. 2).

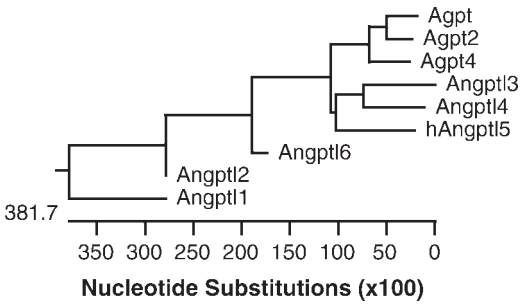

Fig. 1. Phylogenetic tree of angiopoietin and angiopoietin-like family of proteins. Three angiopoietins (Angpt, Angpt2, and Angpt4) and six angiopoietin-like proteins (Angptl1 to Angptl6) are known. Angiopoietin-like protein 5 (hAngptl5) has been cloned from humans only. Nomenclature was adopted from NCBI. Sequence alignment was performed using DNAStar software.

Both Angptl3 and Angptl4 form higher-order structures by both similar and distinct mechanisms

We reported earlier that Angptl4 forms variably sized oligomers resulting from intermolecular disulfide bond formation at the N-terminal coiled-coil domain (24). However, this region of Angptl3 contains no cysteine residues. Consistent with this observation, nondenaturing SDS-PAGE confirmed that Angptl3 exists as a monomer, as it migrates with indistinguishable mobility under denaturing conditions (**Fig. 3B**, lanes 2 and 5). In contrast, Angptl4 exhibited variable-sized oligomers under nonreducing conditions (Fig. 3B, lane 6). To determine if Angptl3 can form higher molecular weight complexes via

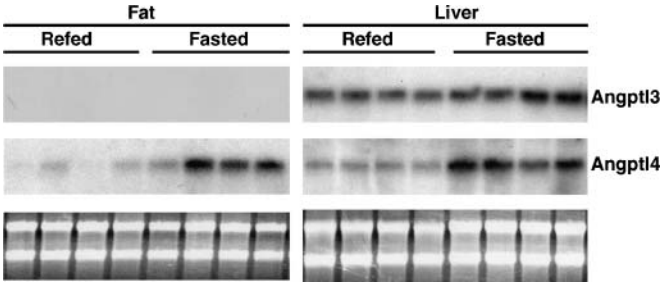

Fig. 2. Angptl4 but not Angptl3 expression is regulated by nutritional status. Male C57BL/6 mice were deprived of food for 24 h and were sacrificed immediately (Fasted) or refed ad libitum for an additional 6 h before sacrifice (Refed). Twenty micrograms of total RNA from liver or 5 μg of total RNA from epididymal fat was analyzed via Northern blot analysis using ^{32}P-labeled cDNA probes for Angptl4 (middle panel) or Angptl3 (upper panel). Approximately equivalent amount of RNA was loaded in each lane, as revealed by ethidium bromide staining of the gel (lower panel). RNA isolated from white adipose tissue and liver of four animals per treatment group was analyzed via Northern blot analysis. Shown is a representative Northern blot (of three independent animal studies).

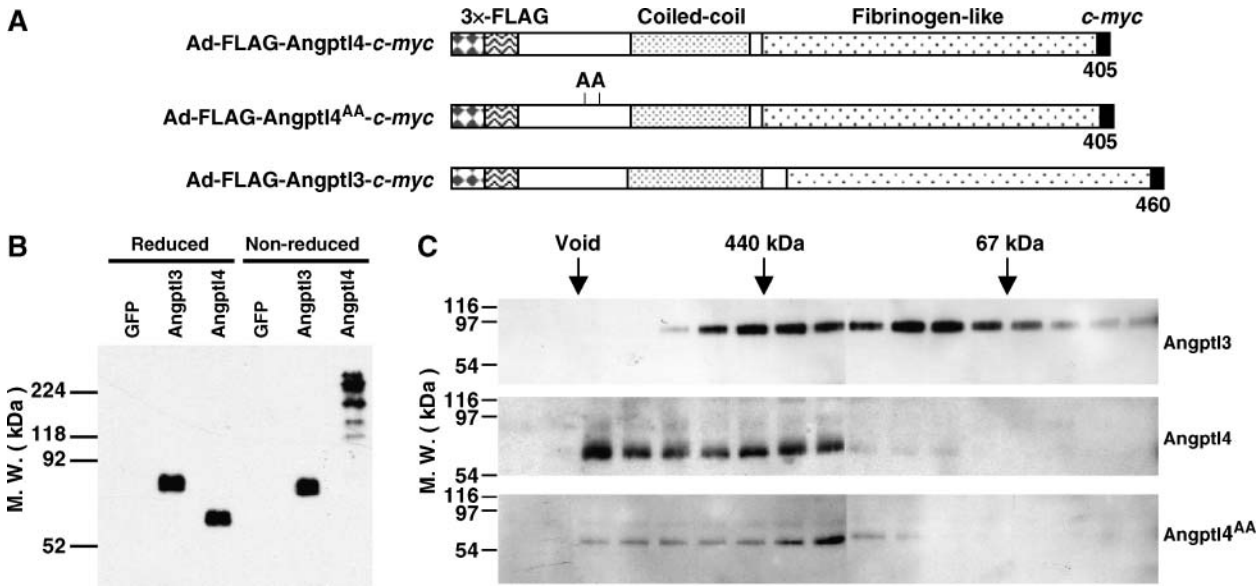

Fig. 3. Angptl3 and Angptl4 have different oligomerization characteristics. (A) Diagram of adenoviral constructs encoding Ad-Flag-Angptl4-*c-myc*, Ad-Flag-Angptl4^{AA}-*c-myc* and Ad-Flag-Angptl3-*c-myc*. Numbers below each construct denote the last residue of each protein. Each region of Angptl3 or Angptl4 is denoted on the top of the diagram. (B) Migration of Angptl3 or Angptl4 under reducing or nonreducing conditions in SDS-PAGE. Samples loaded are viral supernatant of HEK293 cells infected with adenoviruses encoding green fluorescent protein (control), Angptl3, or Angptl4. Proteins are detected by Flag-HRP antibody. Molecular mass markers are indicated on the left. (C) Elution profile of adenovirally produced Angptl3, Angptl4, or Angptl4^{AA} (C76A/C80A mutant) using a Superdex 200 10/300 GL gel filtration column. Angptl4 elutes as high molecular weight oligomers, whereas Angptl3 and Angptl4^{AA} also elute as high molecular weight oligomers but are smaller than Angptl4. Elution positions of molecular markers are marked by arrows.

noncovalent interactions, we performed size-exclusion chromatography. Similar to Angptl4, Angptl3 also elutes as a broad peak but appears to form a smaller complex compared with Angptl4 (Fig. 3C). Interestingly, when both cysteine residues responsible for Angptl4 oligomerization are mutated to alanines, the resulting mutant, Angptl4^{AA}, also elutes as a broad peak and forms complexes (Fig. 3C). These results demonstrate that both Angptl3 and Angptl4 can form higher-order structures in the absence of intermolecular disulfide bond. The presence of cysteine residues causes the formation of larger oligomers and may stabilize such macromolecular structures in vivo, thus prolonging protein half-life in circulation. This is consistent with our observations of the enhanced activity of Angptl4 when it contained both cysteine residues for intermolecular disulfide bond formation (4).

The coiled-coil domain of Angptl4 inhibits LPL activity expressed in C2C12 cells

To determine the minimal structural requirements of Angptl4 necessary for LPL inhibition, we used a muscle-derived cell line that stably expresses human LPL, C2C12-LPL cells (22). We then infected these cells with adenoviruses encoding full-length Angptl4 as well as its N-terminal or C-terminal halves and assessed LPL activity from these cells. Cells that are not transfected with LPL cDNA exhibit low LPL activity, measured by FFA release after incubation with Intralipid (*CTRL*) (**Fig. 4A**). C2C12-LPL cells, untreated or treated with Ad-GFP, exhibit robust LPL activity that is more than 10-fold higher than control cells. Full-length Angptl4 and its N-terminal coiled-coil domain, but not its C-terminal fibrinogen-like domain, reduced Intralipid-derived FFA to basal levels (Fig. 4A). These results are consistent with our prior reports that in vivo, the N-terminal coiled-coil domain of Angptl4 causes hypertriglyceridemia (4).

Interestingly, while Angptl3 mutations in mice are associated with reduced plasma TG levels in a mouse strain that is normally hyperlipidemic and ectopic ANGPTL3 causes elevation of plasma TG levels (1, 2), Angptl3 was unable to inhibit hydrolysis of Intralipid appreciably by C2C12-LPL cells (Fig. 4A). This observation holds true even when Angptl3-expressing C2C12-LPL cells were incubated with Intralipid for different lengths of time, ranging from 2 to 8 h (Fig. 4B). During this period, significant inhibition of LPL activity by Angptl4-expressing cells was observed at all time points. Western blotting analysis showed that a similar amount of Angptl3 and Angptl4 protein was detected in the supernatant of these cells (data not shown), excluding the possibility that recombinant Angptl4 is less stable than Angptl4. To address this issue further, we also performed incubation of adenovirally expressed recombinant Angptl3 and Angptl4 with commercially available LPL to determine the potency of each in inhibiting LPL activity. However, for reasons that are unknown, even Angptl4, which is a very potent inhibitor of LPL and was reported to be more potent than Angptl3 in inhibiting LPL (3), failed to inhibit LPL activity appreciably in such an assay (data not shown).

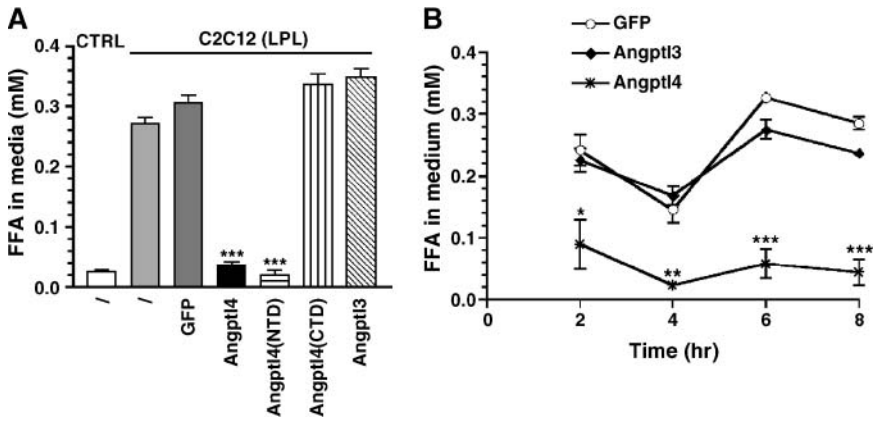

Fig. 4. Inhibition of FFA release by the N-terminal coiled-coil domain of Angptl4. (A) C2C12 cells (*CTRL*) or C2C12 cells stably expressing LPL (*C2C12-LPL*) were untreated (-), or treated with adenoviruses encoding green fluorescent protein, Angptl4, the N-terminal coiled-coil domain of Angptl4 (*Angptl4(NTD)*), the carboxyl fibrinogen-like domain of Angptl4 (*Angptl4(CTD)*),or Angptl3. Cells were infected with these adenoviruses individually for 48 h before Intralipid was added as described in Materials and Methods. Media FFA concentrations were measured 6 h later and corrected for spontaneous FFA release from substrate at 37°C. (B) Media FFA concentrations of C2C12-LPL cells infected with adenoviruses encoding GFP, Angptl3, or Angptl4 were measured at different incubation time points (2, 4, 6, and 8 h) in the presence of Intralipid. Experiments were performed in quadruplicate and repeated at least two times. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$.

Differential induction of Angptl3 and Angptl4 by nuclear receptor agonists

It has been reported previously that expression of Angptl4 is induced by ligands for PPARα and PPARγ (7, 8), while expression of Angptl3 is induced by LXR agonists (5, 6). Because both Angptl3 and Angptl4 raise plasma TG levels, we determined if agonists for these nuclear receptors have any overlapping role in activating Angptl3 and Angptl4 transcription. We treated a liver-derived cell line, FAO cells, as well as C57/Bl6 mice with agonists for PPARα, PPARγ, PPARδ/β, and LXR. In agreement with earlier reports, Angptl3 mRNA levels were increased by approximately 3-fold through treatment with the LXR ligand T1317 (5, 6). However, ligands for neither PPARα nor PPARγ changed the mRNA levels of Angptl3 (**Fig. 5A**). Unexpectedly, the PPARδ/β agonist GW0742 caused a significant decrease of Angptl3 mRNA levels in FAO cells (Fig. 5A).

In contrast to the expression profile of Angptl3, expression of Angptl4 in FAO cells is induced by up to 100-fold by both agonists for PPARα and PPARδ/β (Fig. 5B). Pioglitazone, which activates PPARγ, caused a modest but significant induction of Angptl4 mRNA levels in these cells, while LXR agonists had no effect on Angptl4 mRNA levels (Fig. 5B).

To extend these observations further in vivo, we treated mice with the same set of agonists by oral gavage. In agreement with Northern blotting results (Fig. 2), Angptl3 ex-

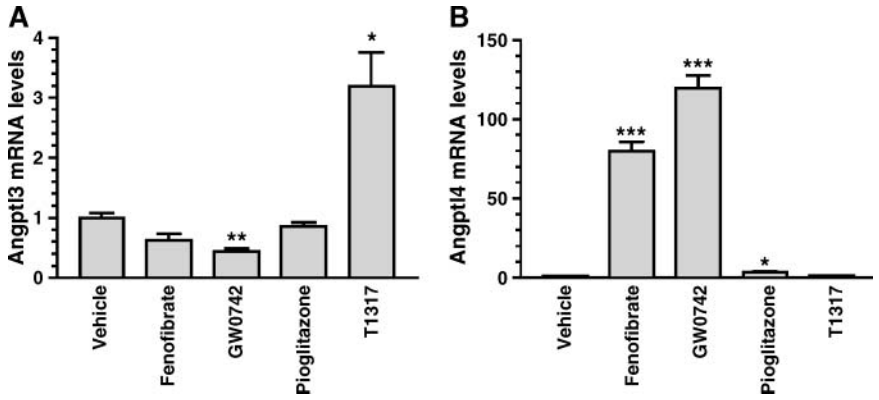

Fig. 5. Regulation of Angptl3 and Angptl4 expression by nuclear receptor agonists in vitro. FAO cells, a rat hepatoma-derived cell line, were treated for 24 h with agonists for PPARα (fenofibrate, 20 μM), PPARβ/δ (GW0742, 5 μM), PPARγ (pioglitazone, 5 μM), or LXR (T1317, 1 μM). mRNA levels of Angptl3 (A) and Angptl4 (B) were determined via quantitative real-time PCR analysis. Samples were prepared in triplicate and experiments were repeated at least twice. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$.

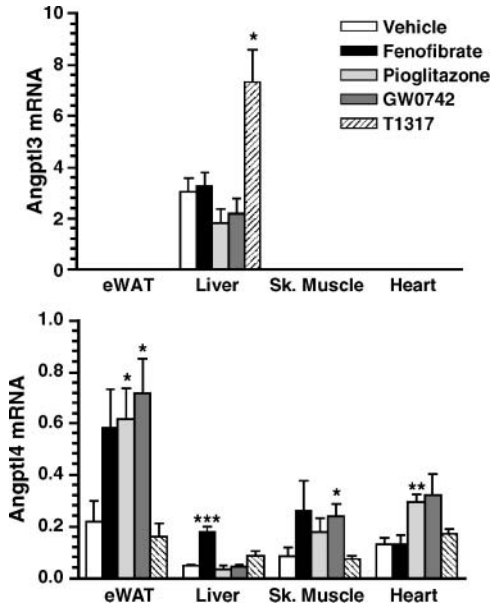

Fig. 6. Regulation of Angptl3 and Angptl4 expression by nuclear receptor agonists in vivo. C57BL/6 mice were treated with agonists for different nuclear receptors by oral gavage for three days. The agonists used are the same as that in Fig. 5. Tissues were harvested 3 h after the last dose. Skeletal muscle (Sk. muscle) was taken from the gastrocnemius muscle. eWAT = epididymal fat. RNA isolation and real-time PCR were performed as described in Materials and Methods. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$ (n = 4 mice/group).

pression is detected only in the liver by quantitative real-time PCR (**Fig. 6A**). Similar to that in FAO cells, only LXR agonists caused an approximately 3-fold elevation of Angptl3 mRNA levels, suggesting that the regulation of Angptl3 mRNA is mediated by direct action of LXR in the liver. In contrast to Angptl3, Angptl4 expression is more widespread. Its mRNA was detected in all four tissues examined (epididymal fat, liver, skeletal muscle, and heart) without agonist treatment and became further induced to various degrees by agonists for PPARα, PPARδ/β, and PPARγ, but not an agonist for LXR (Fig. 6B). Interestingly, changes of Angptl4 mRNA levels in the liver induced by PPARα or PPARβ/δ agonists are much less than that achieved in FAO cells, suggesting that the in vivo regulation of Angptl4 is more complex, possibly involving additional regulatory inputs, such as nutritional status and other as-yet unidentified factors.

DISCUSSION

In this report, we systematically compared the structure and activities of Angptl3 and Angptl4 as well as the regulation of their expression. This study address an important issue on the potential separate roles played by each protein in vivo as well as the induction of each by nuclear hormone receptors, even though both can inhibit LPL activity and raise plasma TG levels. Our results show that these two proteins are regulated differently by nutritional status or ligands for nuclear receptors. Angptl3, which does not form intermolecular disulfide bonds, does in fact form higher-order structures, as assessed via gel filtration chromatography. These results suggest that while both Angptl3 and Angptl4 can inhibit LPL-mediated hydrolysis of lipoproteins, the physiological activities of these angiopoietin-like proteins are not identical.

We were surprised to observe that Angptl3 does not inhibit LPL activity in the C2C12 cell line stably expressing LPL (Fig. 5). Because the role of Angptl3 in plasma TG metabolism is already well established from the study of naturally occurring mouse mutations (1), our results using an LPL-expressing cell line raise the possibility that other lipases, such as the recently identified endothelial lipase (25, 26), might also be a target of Angptl3 and are inhibited by Angptl3, thus raising plasma TG levels. This conjecture is also supported by the observation that Angptl4 is much more potent in raising plasma TG levels (3). Alternatively, adenovirally produced Angptl3 protein may not mimic the condition of its expression in vivo, which could affect its targeting to LPL. The possibility also remains that murine Angptl3 might not inhibit human LPL as potently as Angptl3 of human origin. Additional experiments are needed to clarify these issues.

The regulation of Angptl4 expression by the PPARδ/β agonist GW0742 is intriguing. While Angptl4 mRNA levels are in general increased by treatment with agonists for PPARα and PPARγ, as is shown in Fig. 6 and earlier reports (7, 8), PPARδ/β agonist in some cases increases, while in other cases decreases Angptl4 mRNA levels. For example, treatment of second-passage keratinocytes isolated from newborn foreskins with the PPARδ/β activator GW1514 at 8 μM for 24 h increased Angptl4 mRNA levels by more than 12-fold (10). On the other hand, in a study using PPARδ/β knockout mice, Angptl4 mRNA levels were increased during high-fat feeding of PPARδ/β knockout mice, suggesting that PPARδ/β can also suppress Angptl4 expression (9). However, the fold induction of Angptl4 mRNA in tissues varies, possibly due to the individual variations among the mice used as well as the limited sample size. Further studies are required to delineate the molecular mechanisms underlying such unique patterns of regulation of Angptl4 expression.

Our study further highlights the importance of Angptl4 in fuel partitioning into tissues as its expression is induced in multiple sites, such as heart and skeletal muscle, where LPL action is required to hydrolyze circulating lipoproteins as fuel. Consistent with this notion, we found that transgenic overexpression of Angptl4 in the heart inhibits lipoprotein-derived FFA utilization in cardiac, but not other tissues, via its inhibition of LPL activity (19). This and other earlier studies suggest that the effect of agonists for nuclear receptors on glucose metabolism could have been partially mediated by changes on lipoprotein utilization by Angptl4 in insulin-sensitive tissues (19).

In summary, we provide evidence that differential regulation of expression, assembly, and activity of Angptl3 and Angptl4 may form the basis for their divergent physiologi-

cal functions in vivo. Characterization of mice with deletion of Angptl4, currently underway, should allow a more definitive answer regarding the physiological roles played by these proteins in health and disease.

We thank Dr. Roger Unger for many discussions and Dr. Robert Eckel (University of Colorado Health Sciences Center) for the LPL-expressing C2C12 cell line. Supported by grants to JR from American Heart Association, Texas Affiliate (#04651154) and American Diabetes Association (7-04-RA-94), and to CL from NIH (DK60137).

REFERENCES

1. Koishi, R., Y. Ando, M. Ono, M. Shimamura, H. Yasumo, T. Fujiwara, H. Horikoshi, and H. Furukawa. 2002. Angptl3 regulates lipid metabolism in mice. *Nat. Genet.* **30:** 151–157.
2. Shimizugawa, T., M. Ono, M. Shimamura, K. Yoshida, Y. Ando, R. Koishi, K. Ueda, T. Inaba, H. Minekura, T. Kohama, et al. 2002. Angptl3 decreases very low density lipoprotein triglyceride clearance by inhibition of lipoprotein lipase. *J. Biol. Chem.* **277:** 33742–33748.
3. Yoshida, K., T. Shimizugawa, M. Ono, and H. Furukawa. 2002. Angiopoietin-like protein 4 is a potent hyperlipidemia-inducing factor in mice and inhibitor of lipoprotein lipase. *J. Lipid Res.* **43:** 1770–1772.
4. Ge, H., G. Yang, X. Yu, T. Pourbahrami, and C. Li. 2004. Oligomerization state-dependent hyperlipidemic effect of angiopoietin-like protein 4. *J. Lipid Res.* **45:** 2071–2079.
5. Inaba, T., M. Matsuda, M. Shimamura, N. Takei, N. Terasaka, Y. Ando, H. Yasumo, R. Koishi, M. Makishima, and I. Shimomura. 2003. Angiopoietin-like protein 3 mediates hypertriglyceridemia induced by the liver x receptor. *J. Biol. Chem.* **278:** 21344–21351.
6. Kaplan, R., T. Zhang, M. Hernandez, F. X. Gan, S. D. Wright, M. G. Waters, and T. Q. Cai. 2003. Regulation of the angiopoietin-like protein 3 gene by lxr. *J. Lipid Res.* **44:** 136–143.
7. Kersten, S., S. Mandard, N. S. Tan, P. Escher, D. Metzger, P. Chambon, F. J. Gonzalez, B. Desvergne, and W. Wahli. 2000. Characterization of the fasting-induced adipose factor fiaf, a novel peroxisome proliferator-activated receptor target gene. *J. Biol. Chem.* **275:** 28488–28493.
8. Yoon, J. C., T. W. Chickering, E. D. Rosen, B. Dussault, Y. Qin, A. Soukas, J. M. Friedman, W. E. Holmes, and B. M. Spiegelman. 2000. Peroxisome proliferator-activated receptor gamma target gene encoding a novel angiopoietin-related protein associated with adipose differentiation. *Mol. Cell. Biol.* **20:** 5343–5349.
9. Akiyama, T. E., G. Lambert, C. J. Nicol, K. Matsusue, J. M. Peters, H. B. Brewer, Jr., and F. J. Gonzalez. 2004. Peroxisome proliferator-activated receptor {beta}/{delta} regulates very low density lipoprotein production and catabolism in mice on a Western diet. *J. Biol. Chem.* **279:** 20874–20881.
10. Schmuth, M., C. M. Haqq, W. J. Cairns, J. C. Holder, S. Dorsam, S. Chang, P. Lau, A. J. Fowler, G. Chuang, A. H. Moser, et al. 2004. Peroxisome proliferator-activated receptor (ppar)-beta/delta stimulates differentiation and lipid accumulation in keratinocytes. *J. Invest. Dermatol.* **122:** 971–983.
11. Inukai, K., Y. Nakashima, M. Watanabe, S. Kurihara, T. Awata, H. Katagiri, Y. Oka, and S. Katayama. 2004. Angptl3 is increased in both insulin-deficient and -resistant diabetic states. *Biochem. Biophys. Res. Commun.* **317:** 1075–1079.
12. Shimamura, M., M. Matsuda, Y. Ando, R. Koishi, H. Yasumo, H. Furukawa, and I. Shimomura. 2004. Leptin and insulin down-regulate angiopoietin-like protein 3, a plasma triglyceride-increasing factor. *Biochem. Biophys. Res. Commun.* **322:** 1080–1085.
13. Kim, I., H. G. Kim, H. Kim, H. H. Kim, S. K. Park, C. S. Uhm, Z. H. Lee, and G. Y. Koh. 2000. Hepatic expression, synthesis and secretion of a novel fibrinogen/angiopoietin-related protein that prevents endothelial-cell apoptosis. *Biochem. J.* **346:** 603–610.
14. Backhed, F., H. Ding, T. Wang, L. V. Hooper, G. Y. Koh, A. Nagy, C. F. Semenkovich, and J. I. Gordon. 2004. The gut microbiota as an environmental factor that regulates fat storage. *Proc. Natl. Acad. Sci. U.S.A.* **101:** 15718–15723.
15. Wiesner, G., B. A. Morash, E. Ur, and M. Wilkinson. 2004. Food restriction regulates adipose-specific cytokines in pituitary gland but not in hypothalamus. *J. Endocrinol.* **180:** R1–R6.
16. Belanger, A. J., H. Lu, T. Date, L. X. Liu, K. A. Vincent, G. Y. Akita, S. H. Cheng, R. J. Gregory, and C. Jiang. 2002. Hypoxia up-regulates expression of peroxisome proliferator-activated receptor gamma angiopoietin-related gene (pgar) in cardiomyocytes: role of hypoxia inducible factor 1alpha. *J. Mol. Cell. Cardiol.* **34:** 765–774.
17. Yoshida, K., M. Ono, R. Koishi, and H. Furukawa. 2004. Characterization of the 5′ regulatory region of the mouse angiopoietin-like protein 4. *Vet. Res. Commun.* **28:** 299–305.
18. Mandard, S., F. Zandbergen, N. S. Tan, P. Escher, D. Patsouris, W. Koenig, R. Kleemann, A. Bakker, F. Veenman, W. Wahli, et al. 2004. The direct ppar target fiaf/pgar/angptl4 is present in blood plasma as a truncated protein that is increased by fenofibrate treatment. *J. Biol. Chem.* **279:** 34411–34420.
19. Yu, X., S. C. Burgess, H. Ge, K. K. Wong, R. H. Nassem, D. J. Garry, A. D. Sherry, C. R. Malloy, J. P. Berger, and C. Li. 2005. Inhibition of cardiac lipoprotein utilization by transgenic overexpression of angptl4 in the heart. *Proc. Natl. Acad. Sci. U.S.A.* **102:** 1767–1772.
20. Huang, L., Z. Wang, and C. Li. 2001. Modulation of circulating leptin levels by its soluble receptor. *J. Biol. Chem.* **276:** 6343–6349.
21. Kurrasch, D. M., J. Huang, T. M. Wilkie, and J. J. Repa. 2004. Quantitative real-time polymerase chain reaction measurement of regulators of g-protein signaling mrna levels in mouse tissues. *Methods Enzymol.* **389:** 3–15.
22. Schlaepfer, I. R., L. K. Pulawa, L. D. Ferreira, D. E. James, W. H. Capell, and R. H. Eckel. 2003. Increased expression of the snare accessory protein munc18c in lipid-mediated insulin resistance. *J. Lipid Res.* **44:** 1174–1181.
23. Ward, N. L., and D. J. Dumont. 2002. The angiopoietins and tie2/tek: Adding to the complexity of cardiovascular development. *Semin. Cell Dev. Biol.* **13:** 19–27.
24. Ge, H., G. Yang, L. Huang, D. L. Motola, T. Pourbahrami, and C. Li. 2004. Oligomerization and regulated proteolytic processing of angiopoietin-like protein 4. *J. Biol. Chem.* **279:** 2038–2045.
25. Kratky, D., R. Zimmermann, E. M. Wagner, J. G. Strauss, W. Jin, G. M. Kostner, G. Haemmerle, D. J. Rader, and R. Zechner. 2005. Endothelial lipase provides an alternative pathway for ffa uptake in lipoprotein lipase-deficient mouse adipose tissue. *J. Clin. Invest.* **115:** 161–167.
26. Jin, W., J. S. Millar, U. Broedl, J. M. Glick, and D. J. Rader. 2003. Inhibition of endothelial lipase causes increased hdl cholesterol levels in vivo. *J. Clin. Invest.* **111:** 357–362.