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Angiopoietin-like protein 4 is a potent hyperlipidemia-inducing factor in mice and inhibitor of lipoprotein lipase

Kenichi Yoshida,[1,*] Tetsuya Shimizugawa,[†] Mitsuru Ono,[*] and Hidehiko Furukawa[2,*]

Biomedical Research Laboratories* and Pharmacology and Molecular Biology Research Laboratories,† Sankyo Co., Ltd., 1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140-8710, Japan

Abstract Studies with KK/San, obese and diabetic model mice having a unique hypotriglyceridemia phenotype, revealed that angiopoietin-like protein 3 (ANGPTL3) regulates lipid metabolism in mice. To determine the lipid-modulating role of other ANGPTLs, we focused on ANGPTL4, which overall shows a significant similarity to ANGPTL3. Surprisingly, an intravenous injection of the ANGPTL4 protein in KK/San mice rapidly increased the circulating plasma lipid levels at a higher rate than ANGPTL3 protein. Furthermore, the ANGPTL4 protein inhibited the lipoprotein lipase activity in vitro.—Yoshida, K., T. Shimizugawa, M. Ono, and H. Furukawa. **Angiopoietin-like protein 4 (ANGPTL4) is a potent hyperlipidemia-inducing factor in mice and inhibitor of lipoprotein lipase. *J. Lipid Res.* 2002.** 43: **1770–1772.**

Supplementary key words KK/San • intravenous injection • plasma triglyceride

Angiopoietin-like proteins, ANGPTL3 and ANGPTL4, have been well characterized as secretory proteins. ANGPTL4 has been shown to be a downstream target of peroxisome proliferator-activated receptor-gamma (PPARγ) and possibly has a role in the regulation of systemic lipid metabolism or glucose homeostasis since PPAR target genes identified so far are largely involved in fatty acid metabolism (1). ANGPTL4 could also be a novel endocrine signal involved in the regulation of metabolism, especially under fasting conditions, as demonstrated by the fact that ANGPTL4 mRNA is strongly up-regulated during fasting in white adipose tissue and liver (2). Nevertheless, we still do not know the exact role of the ANGPTL4 protein.

Studies with KK/San mice that model obesity and diabetes but with significantly lower plasma lipid levels have shown that a functional loss of ANGPTL3 at the hypolipidemia (*hypl*) locus leads to abnormally low plasma lipid levels (3). Overexpression of the ANGPTL3 protein in KK/San mice reverses the hypolipidemia phenotype (3). These findings incited us to examine whether other ANGPTLs have an up-regulating effect on lipid metabolism.

Here, we report a novel lipid modulating function of ANGPTL4, the closest resembling protein of ANGPTL3. The ANGPTL4 protein potently increased plasma lipid levels in mice. This unique functional feature of ANGPTL4 is quite similar to that of ANGPTL3 (3). The ANGPTL4 protein also inhibited lipoprotein lipase (LPL) activity in vitro. We considered that ANGPTL4 and ANGPTL3 are functionally unique members of the angiopoietins.

MATERIALS AND METHODS

Vector construction

The cDNA clones used for human ANGPTL4 (GenBank Accession No. BF337379) were obtained from IMAGE consortium. The primers used to amplify full-length human ANGPTL4 (26–406 aa) were 5′-TCCCCCGGGGGACCCGTGCAGTCCAAG-3′ as the sense primer and 5′-CCGCTCGAGGGAGGCTGCCTCTGCTGC-3′ as the anti-sense primer. PCR was performed with 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s. The PCR products were digested with *Sal*I/*Xho*I and cloned into the corresponding site of pGEX-4T-2 (Amersham). All constructs were confirmed by sequencing. Several independent clones were tested for their protein expression level and the most suitable clone was used for a larger preparation of the protein.

Preparation of GST fusion proteins

The protein was expressed in *Escherichia coli* BL21CodonPlus (DE3)-RIL strain (Stratagene) by induction of log phase cells (O.D.600 = 0.5–0.7) with 1 mM isopropyl-1-thio-β-D-galactopyranoside at 37°C for 4 h. The cell pellet was collected and resuspended in ice-cold sonication buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol) following sonication using Branson sonifier 250 and Triton X-100, was added up

Manuscript received 31 July 2002.
Published, JLR Papers in Press, September 16, 2002.
DOI 10.1194/jlr.C200010-JLR200

[1] Present address: Genetic Diagnosis, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639.
[2] To whom correspondence should be addressed.
e-mail: furuka@shina.sankyo.co.jp

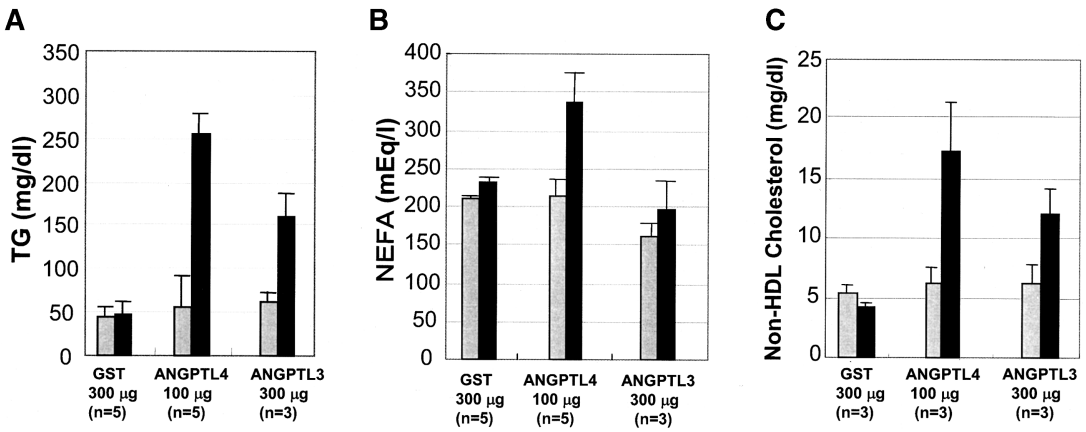

Fig. 1. Lipid-modulating activities of ANGPTL4 in mice. Each protein (100 μg or 300 μg) was administrated intravenously through the tail vein to KK/San mice. Before (time 0; gray bar) and at 30 min after injection (black bar), plasma was obtained and triglyceride (A), NEFA (B), and non-HDL cholesterol (chylomicron, VLDL and LDL fraction) (C) levels were measured for GST control, GST-ANGPTL4 (26–406 aa), and recombinant full-length ANGPTL3 protein. Results represent the mean ± SD of the indicated number of animals.

to 1%. The pellet was washed repeatedly with washing buffer (0.5% Triton X-100, 1 mM EDTA), passed through a Kurimover resin column (Kurita Water Industries Ltd.), and then suspended in 8 M urea for 1 h at room temperature. The supernatant was collected and dialyzed against 4 M urea for 1 h at 4°C, 2M urea for 1 h at 4°C, sonication buffer for 1 h at 4°C, and left overnight at 4°C. The supernatant of the dialyzed fraction was directly added to glutathione-Sepharose 4B (Amersham) and eluted with 10 mM of reduced glutathion (pH 8.0). Each fraction was subjected to SDS-PAGE followed by staining with Coomassie brilliant blue and dialyzed against 1×PBS (pH 7.6) overnight at 4°C. Protein concentration was determined by the Bradford assay method using BSA (Sigma) as a standard (Bio-Rad).

Preparation of LPL

Rat white adipose precursor cells were purchased (Hokudo Co., Ltd.) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in a 25 cm^2 culture flask. To prepare the LPL sample, cells were cultured with DMEM supplemented with 10 μg/ml insulin and 2.5 μM dexamethasone for 48 h. The medium was changed to DMEM supplemented with 10 μg/ml insulin and incubated for 2–3 days. Then, 2 ml of DMEM supplemented with 50 ng/ml insulin and 10 U/ml heparin sodium was added and the cells were incubated for 1 h. The supernatant of the culture medium was used for further assay.

LPL assay

An LPL assay was performed as described previously (4). Briefly, the supernatant containing LPL (100 μl) was mixed with an equal amount of substrate solution (2 mM glycerol-Tri[9, 10 (n)-^{3}H]) oleic acid (131 Kbeq/μmol, Amersham), 0.189 μg/ml L-α-phosphatidylcholine (Sigma), 14 mg/ml bovine serum albumin (Sigma), 140 mM Tris-HCl (pH 8.0), 15% (v/v) glycerol, and 10% (v/v) heat-inactivated fetal calf serum. Then, the protein sample was added and incubated for 2 h at 37°C. The reaction was stopped by vortex mixing 1.05 ml of 0.1 M K_2CO_3-Boric acid buffer (pH 10.5) and 3.25 ml of methanol-chloroform-hexane (141:125:100, v/v/v), and then the protein was isolated by centrifugation (3,000 *g* for 15 min). Radioactivity in an aqueous/methanol fraction containing free ^{3}H was measured using a liquid scintillation counter (Beckman). One unit of LPL activity was determined as the amount required to produce 1 μmol of fatty acid per min. The results were obtained from three determinations and expressed as a percent of the control (3.7×10^{-7} M of GST).

Animal assay

KK/San mice were obtained as described previously (3). Recombinant proteins (100 μg or 300 μg) were intravenously injected into male KK/San mice (13–15 weeks). Before and at 30 min after injection, the plasma was recovered and plasma triglyceride, non-esterified fatty acid (NEFA), and total cholesterol levels were measured by commercial kits (Wako Pure Chemical Industries). The non-HDL cholesterol (non-HDL-C) level was determined by HPLC.

RESULTS

ANGPTL4 increase of plasma lipid level in vivo

Identification of ANGPTL3 as a lipid-modulating protein in mice prompted us to test ANGPTL3 homologous protein (3). The homology and phylogenetic tree based on CLUSTALW analysis enables us to search the closest protein of ANGPTL3. It was revealed that ANGPTL3 is the

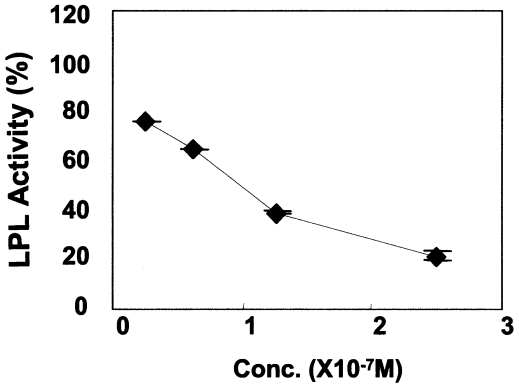

Fig. 2. Inhibitory activities of ANGPTL4 on lipoprotein lipase. Human ANGPTL4 (26–406 aa) was produced as a GST fusion protein in *Escherichia coli* and the indicated concentrations of purified protein (1, 2.5, 5, and 10 μg/ml, respectively) were used for the lipoprotein lipase assay. Results are expressed as a percent of the control (3.7×10^{-7} M of GST) and represent the mean ± SD of three determinations.

closest relative to ANGPTL4. Therefore, we administrated the ANGPTL4 protein into KK/San mice intravenously and measured the plasma triglyceride, non-esterified fatty acid (NEFA) and total cholesterol levels. We used the recombinant ANGPTL3 protein that was purified from mammalian cells (3) as a positive control and GST as a negative control. GST did not show any change of lipid content in plasma (**Fig. 1**). In contrast to GST, the ANGPTL4 protein significantly increased the triglyceride level in plasma by about 3- to 5-fold 30 min after injection (Fig. 1A).

In addition to the effect on plasma triglyceride levels, ANGPTL4 rapidly increased the NEFA level in plasma (Fig. 1B). However, it had no significant effect on the level of total cholesterol (the mean ± SD for plasma total cholesterol was 91.1 ± 8.04 → 65.0 ± 6.25 for ANGPTL3 and 86.1 ± 5.06 → 69.1 ± 8.38 for ANGPTL4, respectively). Further analysis revealed that the higher level of plasma triglyceride after ANGPTL4 protein injection is mainly due to an increase in the fraction of non-HDL-C including chylomicrons and very low-density lipoproteins (VLDL) (Fig. 1C). More importantly, the in vivo activity of ANGPTL4 was higher than that of ANGPTL3, which raised the plasma triglyceride level by about 2- to 3-fold (Fig. 1A). Thus, our results strongly suggest that ANGPTL4 and ANGPTL3 are functionally unique angiopoietin-related proteins due to their lipid-modulating effect in mice.

ANGPTL4 decrease of lipoprotein lipase activity in vitro

In KK/San mice whose ANGPTL3 locus was mutated, LPL activity in epididymal adipose tissue was 2-fold higher than that in KK mice, which have wild-type ANGPTL3 (3). Furthermore, ANGPTL3 has been shown to inhibit LPL activity in vitro (5). To further demonstrate that ANGPTL4 has a lipid-modulating effect, we investigated the relationship between LPL activity and ANGPTL4 by establishing a GST-fusion protein and using it in an LPL assay (4). The results showed that the ANGPTL4 protein potently inhibited the LPL activity with an IC_{50} of 0.2×10^{-7} M (**Fig. 2**). This observation corresponded well with the results obtained in the study examining the in vivo lipid-modulating action of ANGPTL4. On the other hand, recombinant human angiopoietin-2 (Techne) and GST were unable to inhibit the LPL activity (data not shown).

DISCUSSION

By comparing the sequence homology and phylogenetic tree within the database, we postulated that ANGPTL4, the closest resembling protein of ANGPTL3, could be exerting a lipid-modulating function similar to ANGPTL3 (3). To support this idea, we intravenously administrated ANGPTL4 protein in KK/San mice and measured the plasma lipid content 30 min after injection, since the time-course analysis following recombinant ANGPTL3 protein administration demonstrated a rapid triglyceride-increasing effect and attainment of peak NEFA plasma levels after 1 h (3). Based on the lipid-modulating function found for ANGPTL4 and its rapid and potent onset of activity, it was considered that ANGPTL4 increases plasma triglyceride levels by inhibiting LPL activity. To further confirm this hypothesis, we conducted an LPL assay and verified the ANGPTL4 effect on LPL. LPL is known as a key enzyme in lipid metabolism, hydrolyzing plasma triglyceride from chylomicrons and VLDL. It also controls the fatty acid uptake into tissues (6). Remarkably, the ANGPTL4 showed a significant inhibition of LPL activity.

The redundant function of ANGPTL4 and ANGPTL3 may reflect the clinical importance of these proteins in lipid metabolism. Earlier works identified ANGPTL4 as a downstream target of PPARα, the predominant form in the liver, playing a pivotal role in the regulation of lipid metabolism (2). Interestingly, ANGPTL4 was also demonstrated to be a PPARγ target gene and expressed highly in adipose tissue, although ANGPTL3 is the most predominantly expressed enzyme in the liver (1, 7). Despite the redundant lipid-modulating function identified for proteins ANGPTL4 and ANGPTL3, the tissue distribution is different. Further characterization of the transcriptional regulation of ANGPTL4 and ANGPTL3 will be helpful in understanding which gene is important for different clinical settings.

In conclusion, we demonstrated that ANGPTL4 is a potent hyperlipidemia-inducing factor in mice and that this activity is most likely induced by the inhibition of LPL activity. This finding raises an interesting possibility that ANGPTL4, in addition to ANGPTL3, might prove to be novel targets for therapeutic agents for patients who suffer from hyperlipidemia.

The authors thank Dr. Koishi, Dr. Inaba, and Mr. Shimamura for their helpful advice.

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