Muscle-Derived Angiopoietin-Like Protein 4 is Induced by Fatty Acids via PPARδ and is of Metabolic Relevance in Humans

Harald Staiger, PhD1, Carina Haas1, Jürgen Machann2, Roman Werner1, Melanie Weiss1, Fritz Schick, MD, PhD2, Fausto Machicao, PhD1, Norbert Stefan, MD1, Andreas Fritsche, MD1, Hans-Ulrich Häring, MD1

1 Department of Internal Medicine, Division of Endocrinology, Diabetology, Angiology, Nephrology, and Clinical Chemistry, Eberhard-Karls-University Tübingen, D-72076 Tübingen, Germany
2 Department of Experimental Radiology, Eberhard-Karls-University Tübingen, D-72076 Tübingen, Germany

Corresponding author:
Dr. rer. nat. Harald Staiger; Internal Medicine IV, Medical Clinic Tübingen, Otfried-Müller-Strasse 10, D-72076 Tübingen, Germany.
E-mail: harald.staiger@med.uni-tuebingen.de

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ABSTRACT

Objective: Long-chain fatty acids (LCFA) contribute to metabolic homeostasis in part via gene regulation. This study’s objective was to identify novel LCFA target genes in human skeletal muscle cells (myotubes).

Research Design and Methods: In vitro methods included culture and treatment of human myotubes and C2C12 cells, gene array analysis, real-time RT-PCR, Western blotting, ELISA, chromatin immunoprecipitation, and RNA interference. Human subjects (two cohorts) were characterized by oral glucose tolerance test, hyperinsulinemic-euglycemic clamp, magnetic resonance imaging and spectroscopy, and standard blood analyses (glucose, insulin, C-peptide, and plasma lipids).

Results: We show here that ANGPTL4 (encoding angiopoietin-like protein 4) represents a prominent LCFA-responsive gene in human myotubes. LCFA activated peroxisome proliferator-activated receptor (PPAR) δ, but not PPARα or γ, and pharmacological activation of PPARδ markedly induced ANGPTL4 production and secretion. In C2C12 myocytes, knock-down of PPARD, but not of PPARG, blocked LCFA-mediated ANGPTL4 induction, and LCFA treatment resulted in PPARδ recruitment to the ANGPTL4 gene. In addition, pharmacological PPARδ activation induced LIPE (encoding hormone-sensitive lipase), and this response crucially depended on ANGPTL4, as revealed by ANGPTL4 knock-down. In a human cohort of 108 thoroughly phenotyped subjects, plasma ANGPTL4 positively correlated with fasting non-esterified fatty acids (p=0.0036) and adipose tissue lipolysis (p=0.0012). Moreover, in 38 myotube donors, plasma ANGPTL4 levels and adipose tissue lipolysis in vivo were reflected by basal myotube ANGPTL4 expression in vitro (p=0.02, both).

Conclusions: ANGPTL4 is produced by human myotubes in response to LCFA via PPARδ, and muscle-derived ANGPTL4 seems to be of systemic relevance in humans.
The metabolic syndrome (MS), a cluster of health problems including visceral obesity, subclinical inflammation, insulin resistance, and type 2 diabetes, is the prevailing metabolic disorder in Western industrialized countries. The syndrome is caused by environmental factors (high-caloric food intake, sedentary lifestyle) combined with a genetic predisposition. Elevated plasma non-esterified fatty acid (NEFA) levels are frequently observed in MS patients and result from increased lipolysis of insulin-resistant white adipose tissue (WAT) and/or chronically excessive dietary fat intake (1).

Among the major plasma long-chain fatty acid (LCFA) species, the saturated fatty acids (SFA) palmitate and stearate are of particular interest with respect to their potential involvement in metabolic disarrangements, such as hyperglycemia, hyperinsulinemia, hypertriglyceridemia, and β-cell dysfunction: administered chronically, they reduce muscular glucose disposal (2), promote hepatic triglyceride and VLDL synthesis (3), impair hepatic insulin clearance (4), and inhibit pancreatic insulin secretion (5). One proposed mechanism underlying all these metabolic LCFA effects is ectopic lipid deposition in muscle, liver, and pancreatic islets. The molecular links between LCFA actions and ectopic lipid deposition are however not well understood.

Recent data suggest that SFA exert direct gene regulatory effects and may also in this way contribute to MS (6). We reported that palmitate and stearate, via nuclear factor κB (NF-κB) activation, provoke an inflammatory response in human skeletal muscle (SKM) and coronary artery endothelial cells by induction of the gene encoding interleukin-6 (7;8). Very high concentrations of these LCFA species, again via NF-κB, induce pro-apoptotic genes and promote apoptotic death of human coronary artery endothelial cells (9). Furthermore, SFA impair mitochondrial activity of SKM cells by repression of the gene encoding peroxisome proliferator-activated receptor (PPAR) γ coactivator-1β (10), and reduced muscular oxidative capacity was clearly demonstrated in patients with insulin resistance and type 2 diabetes (11;12). By contrast, unsaturated fatty acids (UFA), such as palmitoleate, oleate, and linoleate, increase mitochondrial activity of SKM cells by induction of PPARγ coactivator-1α (10).

Even though LCFA-regulated transcription factors (NF-κB, PPARs) are known to date, LCFA-dependent gene regulation and its involvement in metabolic disease is not yet well understood. Therefore, it was this study’s objective to identify, in human SKM cells differentiated in vitro (myotubes), novel LCFA target genes that could represent potential candidate contributors to MS.

METHODS SUMMARY

A detailed description is given in the online appendix.

Primary human myotubes and murine C2C12 myocytes were used for cell experiments. Microarray analysis was performed with Affymetrix Human Genome U133 Plus 2.0 arrays. Real-time reverse transcriptase (RT)-PCR was performed with SYBR Green I dye on a LightCycler™. The anti-ANGPTL4 antibody from BioVendor was used for immunoblotting. Intracellular and secreted ANGPTL4 was quantified by ELISA. For RNA interference (RNAi), siGENOME siRNA sets designed by Dharmacon were used. Chromatin immunoprecipitation (ChIP) analysis was performed with the anti-PPARδ antibody K-20 from Santa Cruz Biotechnology.

All 38 myotube donors underwent an oral glucose tolerance test (OGTT) and a hyperinsulinemic-euglycemic clamp (HEC). The 108 subjects with plasma ANGPTL4 activity of SKM cells by repression of the gene encoding peroxisome proliferator-activated receptor (PPAR) γ coactivator-1β (10), and reduced muscular oxidative capacity was clearly demonstrated in patients with insulin resistance and type 2 diabetes (11;12). By contrast, unsaturated fatty acids (UFA), such as palmitoleate, oleate, and linoleate, increase mitochondrial activity of SKM cells by induction of PPARγ coactivator-1α (10).

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results and discussion

Gene array analysis. In an initial attempt to identify novel LCFA target genes related to MS, we treated human myotubes derived from a healthy male German donor with bovine serum albumin (BSA; carrier control), the SFA palmitate, or the UFA linoleate and performed whole-genome gene array analysis. Palmitate treatment repressed 181 and induced 316 genes, linoleate repressed 30 and induced 104 genes. Thus, LCFA appear to influence the expression of numerous genes in human myotubes. Notably, only 5 genes were repressed (Supplementary Table 3) and only 35 genes were induced (Table 1) by both palmitate and linoleate. Thus, the majority of LCFA-regulated genes appear to represent LCFA-specific targets, and modulation of their expression might depend on LCFA chain length and/or the degree of saturation. The gene that revealed highest fold-induction by both palmitate and linoleate was ANGPTL4 (Table 1), encoding angiopoietin-like protein 4 (ANGPTL4). The sole purpose of this single non-replicated experiment was to generate new hypotheses. Therefore, these results cannot be generalized.

ANGPTL4 was described as hepatic fibrinogen/angiopoietin-related protein (13), fasting-induced adipose factor (14), and PPARγ target gene related to angiopoietin (15) and was characterized as a secreted protein predominantly produced by WAT, but also at lower levels by other tissues (13-15). The role of ANGPTL4 was extensively explored in mice by injection, targeted gene knock-out as well as transgenic and retroviral overexpression, and in this way ANGPTL4 was shown to affect lipid metabolism: by inhibition of lipoprotein lipase (LPL), clearance of VLDL and chylomicrons is blocked and hypertriglyceridemia provoked (16-20). Furthermore, ANGPTL4 stimulates WAT lipolysis (21) resulting in elevated plasma glycerol and NEFA levels (16;21). Besides hyperlipidemia, ANGPTL4 promotes WAT weight loss and hepatic steatosis (18;21). Importantly, ANGPTL4 expression was consistently found up-regulated in genetic mouse models of obesity and type 2 diabetes (15). Thus, ANGPTL4 represents a metabolically relevant candidate gene induced by common plasma LCFA species.

Human myotube ANGPTL4 expression before and after LCFA treatment. To assess whether ANGPTL4 is produced by human myotubes at relevant levels, we measured basal ANGPTL4 mRNA expression by real-time RT-PCR. Untreated human myotubes expressed 20.9±7.2 fg ANGPTL4 mRNA/µg total RNA (mean±SD; n=5). This level was not only in the range of that found in a representative human SKM biopsy (12 fg ANGPTL4 mRNA/µg total RNA) but also represents about 40% of the mRNA level found in subcutaneous WAT (56 fg ANGPTL4 mRNA/µg total RNA), a major site of ANGPTL4 expression.

To confirm the gene array results and to explore the influence of other plasma LCFA on ANGPTL4 expression, we treated human myotubes with BSA or selected major plasma LCFA species and subsequently quantified the cellular ANGPTL4 mRNA contents by real-time RT-PCR (normalized to 28S-rRNA). ANGPTL4 mRNA expression was induced 10-50-fold by all LCFA tested (Figure 1A). The ANGPTL4 mRNA levels found after treatment with palmitate, stearate,
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palmitoleate, oleate, linoleate, or a combination of palmitate and linoleate were significantly different from their respective BSA controls (Figure 1A). The ANGPTL4 mRNA contents detected after treatment with a combination of palmitate and linoleate did not significantly differ from those obtained with palmitate or linoleate alone. Thus, treatment with palmitate or linoleate alone might already be sufficient to reach the maximum response of LCFA-inducible ANGPTL4 expression. Moreover, ANGPTL4 induction did neither depend on the chain length nor on the saturated/unsaturated nature of the LCFA, but rather represented a general LCFA effect. To verify these LCFA-mediated gene regulations at the protein level, we used the first commercially available ELISA kit and measured intracellular ANGPTL4 contents after 20-h LCFA treatment. All LCFA increased intracellular ANGPTL4 protein 1.5-2.3-fold with palmitate, oleate, and linoleate reaching statistical significance (Figure 1B). The observed differences between mRNA and protein induction rates are a well-known phenomenon that most probably reflects that gene transcription rate and mRNA half-life are not necessarily tightly linked to translation efficiency and protein half-life. In addition, we tried to measure and visualize secreted ANGPTL4 protein. However, the high BSA concentrations used for LCFA treatment interfered with the detection of secreted ANGPTL4. To see whether human myotube ANGPTL4 expression was modulated by glucose, we performed a dose-response experiment with 2.5, 5, 10, and 25mM glucose (added to glucose-free medium). After 20h, there was no significant glucose effect on basal myotube ANGPTL4 expression (p=0.9; ANOVA; n=4).

Since ANGPTL4 was described as a fasting-induced factor (14;15;22), and plasma LCFA are known to be elevated during the fasting state due to unsuppressed WAT lipolysis, these results turn plasma LCFA into potential candidate mediators of fasting-induced ANGPTL4 production.

Modulation of human myotube PPAR isoform expression and activation by LCFA. ANGPTL4 is a known target gene of PPARα and δ in liver (14;22) and PPARγ in WAT (14;15), and a PPAR response element was identified in intron 3 of the ANGPTL4 gene (23). To see whether LCFA enhance myotube ANGPTL4 expression via induction of the genes encoding PPARα, γ, or δ (PPARA, PPARG, or PPARD, respectively), we quantified the respective mRNA levels prior to and after LCFA treatment. In untreated human myotubes, the PPARD mRNA contents were about 10-fold higher than those of PPARA and PPARG (data not shown). Neither PPARA nor PPARG mRNA contents were significantly altered by LCFA treatment when compared to their respective BSA controls (p≥0.2, both; ANOVA; n=5). Furthermore, all LCFA shown above to induce ANGPTL4 (Figure 1A) did not induce PPARD expression with only one exception: stearate increased PPARD mRNA contents 2.6-fold (p<0.0001; ANOVA; post hoc p<0.05; n=5). This stearate-specific effect however cannot explain the general effect of LCFA on ANGPTL4 expression. To explore whether stearate’s inductive effect is translated to the protein level, we performed immunoblot analysis. None of the LCFA tested, including stearate, provoked relevant changes in PPARδ protein after 20h of treatment (n=3; data not shown). Therefore, we suggest that LCFA act as PPAR ligands or activators (intracellularly metabolized to ligands), but not as regulators of PPAR expression/production.

To examine whether LCFA are able to activate one of the three PPAR isoforms in human myotubes, we also quantified the mRNA levels of PPAR target genes, i.e. PDK4 (encoding pyruvate dehydrogenase kinase 4) as target of PPARδ and α (24),
CD36 (encoding fatty acid translocase) and UCP3 (encoding uncoupling protein 3) as PPARα-specific targets (25;26), and PPARG as target of PPARγ itself (27). Compared to their respective BSA controls, none of the LCFA modulated the expression of CD36, UCP3, or PPARG in these cells (p≥0.4, all; ANOVA; n≥4). By contrast, all LCFA induced PDK4 mRNA expression 7-22-fold, and the effects of palmitate, palmitoleate, oleate, linoleate, and the combination of palmitate and linoleate were statistically significant (Figure 1C). This led us to assume that the LCFA tested in this study selectively activate PPARδ in human myotubes, at least at the concentration tested (0.5mM).

Stimulation of human myotube ANGPTL4 production by pharmacological PPAR activation. To further investigate the role of PPAR isoforms in human myotube ANGPTL4 expression, we treated the cells with the PPARα-specific fibrates Wy-14,643 and fenofibrate, the PPARγ-specific thiazolidinediones troglitazone and rosiglitazone, and the PPARδ-specific activator GW501516. As presented in Figure 2A, ANGPTL4 mRNA expression was markedly induced by GW501516 and moderately induced by troglitazone (17-fold and 4-fold, respectively). By contrast, ANGPTL4 mRNA expression was neither altered by rosiglitazone, a thiazolidinedione with ten-fold higher affinity for PPARγ than troglitazone, nor by any of the fibrates tested. Only GW501516 treatment reached a level of ANGPTL4 induction comparable to that found with LCFA. Furthermore, ANGPTL4 induction by GW501516 followed the same kinetics as the other PPARδ target gene PDK4 (Figures 2B and 2C). We also tested GW501516’s effect on intracellular ANGPTL4 protein contents using ELISA. GW501516 treatment (1µM, 20h) increased intracellular ANGPTL4 protein 2.5-fold over DMSO control (365.6±52.9pg/mg vs. 908.2±154.0 pg/mg; p=0.0158; t-test; n=4). These data strengthened our suggestion of PPARδ being a crucial mediator of LCFA-induced ANGPTL4 expression in human myotubes.

In addition, 48-h treatment of human myotubes with GW501516 resulted in continuous accumulation of ANGPTL4 protein in the culture supernatant (Figure 2D). Hence, human ANGPTL4 can be added to the novel and growing list of muscle-derived secreted proteins with metabolic functions (myokines) which also comprises interleukin-6 (28), interleukin-15 (29), and musclin (30). Besides full-length ANGPTL4 (~70kDa), a 50-kDa C-terminal and a 26-kDa N-terminal fragment, both with biological activity, were reported to circulate in the bloodstream (18;23;31). Therefore, we asked whether ANGPTL4 secreted into the supernatant by GW501516-treated human myotubes is proteolytically cleaved. Immunoblotting revealed that both full-length ANGPTL4 and the C-terminal fragment accumulated in the culture supernatant during the treatment period (Figure 2E). The N-terminal fragment could not be detected with antibodies from different suppliers directed against full-length or the N-terminal part of ANGPTL4 and, thus, seems to be rapidly degraded. In conclusion, ANGPTL4 secreted by human myotubes is cleaved and biologically activated.

Role of PPARδ in LCFA-enhanced ANGPTL4 expression in C2C12 myocytes. To evidence that LCFA-induced myocyte ANGPTL4 expression depends on PPARδ, we knocked down PPARD expression by RNAi. This was done in murine C2C12 myocytes because human myotubes could not be efficiently transfected. First, we treated C2C12 myocytes with representative SFA and UFA species. As presented in Figure 3A, all LCFA tested increased the C2C12 ANGPTL4 mRNA contents when compared to their respective BSA controls, and the effects of stearate, oleate, and linoleate reached the
level of significance. Thus, LCFA-induced ANGPTL4 expression was not restricted to human myotubes, but was reproduced in a murine SKM cell line. Moreover, as in human myotubes, GW501516 treatment induced ANGPTL4 mRNA expression up to 20-fold in C2C12 cells (Figure 3B). As depicted in Figure 3C, transfection with siRNA directed against PPARD, but not with control siRNA directed against bacterial luciferase, reduced C2C12 PPARD mRNA contents by 82%. After PPARD knock-down, oleate-induced ANGPTL4 expression was significantly impaired (2.4-fold in the presence vs. 6.8-fold in the absence of PPARD siRNA), and, moreover, the oleate effect was no longer significant (Figure 3D). Furthermore, control transfection with siRNA directed against bacterial luciferase still allowed marked ANGPTL4 induction by oleate (Figure 3D). Thus, induction of ANGPTL4 expression in C2C12 myocytes by the representative LCFA oleate requires PPARδ. Importantly, C2C12 PPARA expression ranged at the detection limit of the real-time PCR method, even upon utilization of sensitivity-enhancing hybridization probes. Therefore, we conclude that PPARA is not expressed in these cells and is not involved in LCFA-induced ANGPTL4 expression. Transfection of the cells with siRNA directed against PPARG, but not with control siRNA, reduced C2C12 PPARG expression by 88%. However, this manipulation had no significant impact on oleate-induced ANGPTL4 expression (p=1.0; t-test; n=3). Hence, LCFA effects on ANGPTL4 expression are specifically mediated by PPARδ.

To ultimately prove that PPARδ is activated by LCFA and binds to the ANGPTL4 gene, we performed ChiP analysis in C2C12 cells treated for 6h with oleate. In the anti-PPARδ immunoprecipitate, markedly more ANGPTL4 intron 3 DNA (harbouring the PPRE) could be detected in oleate-treated cells as compared to BSA-treated cells (Figure 3E). This demonstrates that PPARδ is activated and recruited to the PPRE of the ANGPTL4 gene upon LCFA treatment.

**Effect of pharmacological ANGPTL4 induction on the expression of C2C12 myocyte lipases.** ANGPTL4 is reported to enhance WAT lipolysis via induction of adipose triglyceride lipase (ATGL) (21). We therefore asked whether triglyceride lipases of SKM cells, which are required for breakdown of intramyocellular triglycerides, are also under the control of muscle-derived ANGPTL4 (mANGPTL4). Untreated C2C12 myocytes expressed 46.3±10.6 fg PNPLA2 mRNA (encoding ATGL) and 11.8±2.0 fg LIPE mRNA (encoding hormone-sensitive lipase, HSL)/µg total RNA (means±SD; n=3). Treatment of C2C12 cells with GW501516 provoked significant increments over time of LIPE and PNPLA2 mRNA (Figures 4A and 4B), as compared to DMSO (carrier control). However, the kinetics of both gene regulations was completely different from that of the aforementioned PPARδ target genes PDK4 and ANGPTL4 in that these lipases showed delayed induction (compare Figures 2B and 2C with Figures 4A and 4B). This suggests that LIPE and PNPL2A are not direct PPARδ target genes.

The GW501516 effect on PNPLA2 expression was only obvious at very late time points (beyond 24h after start of treatment) when DMSO alone also revealed some gene regulatory effects. Using RNAi, we therefore assessed here the potential auto-/paracrine role of myocyte ANGPTL4 expression during the moderately delayed stage of LIPE expression (at 20h of treatment). As depicted in Figure 4C, transfection with siRNA directed against ANGPTL4, but not with control siRNA directed against bacterial luciferase, reduced the GW501516-induced ANGPTL4 mRNA contents by 90%. The ANGPTL4 knock-down significantly impaired GW501516-mediated LIPE induction (1.6-fold in the presence vs. 2.6-fold in the
absence of ANGPTL4 siRNA), and, in addition, the GW501516 effect was no longer significant (Figure 4D). Again, control transfection with bacterial luciferase siRNA did not significantly alter the GW501516 effect on LIPE expression (Figure 4D).

Here, we show that pharmacological PPARδ activation up-regulates LIPE expression in C2C12 SKM cells and provide preliminary evidence for a role of mANGPTL4 in this gene-regulatory event. LIPE induction is supposed to enhance intramyocellular lipolysis and to increase endogenous fatty acyl-CoA, the preferred substrate of SKM oxidative metabolism stimulated by PPARδ agonists (32). Moreover, this finding could point to an auto-/paracrine function of mANGPTL4 in SKM. To corroborate the role of mANGPTL4 in the breakdown of intramyocellular lipids, further studies, e.g., in muscle-specific PPARδ gain- and loss-of-function animal models, are clearly needed. Since C2C12 cells do not store measurable amounts of triglycerides (data not shown), other muscle cell models are required to study ANGPTL4’s lipolytic effect at the cellular level.

**Relationship between plasma ANGPTL4 and metabolic traits in humans**

Since our in vitro data demonstrated a close relationship between ANGPTL4 and lipid metabolism, we assessed this protein’s role in lipid metabolism, insulin sensitivity, and insulin secretion in humans in vivo. To this end, we quantified plasma ANGPTL4 in 108 thoroughly phenotyped participants of the TUEbingen Lifestyle Intervention Program (TULIP) (33-36), a cohort characterized by a wide range of age, BMI, body fat content, insulin sensitivity, and insulin secretion (clinical characteristics presented in Supplementary Table 2) using ELISA.

The mean plasma ANGPTL4 concentration measured was 1.73±0.11ng/ml (mean±SE; range: 0.37-8.00ng/ml). The plasma ANGPTL4 levels were not correlated with gender or age (p=0.9, both), and this is in good agreement with recently published data (37). There were no significant correlations with BMI, waist-hip ratio, body fat measured by bioelectrical impedance, total, visceral, and non-visceral fat mass measured by magnetic resonance imaging, or plasma levels of adiponectin and leptin (p≥0.06, all; after appropriate adjustments). However, stratification of the cohort into lean (BMI<27kg/m²; 19.5-26.9kg/m²; n=30) and obese (BMI≥30kg/m²; 30.0-48.4kg/m²; n=39) subjects revealed significantly elevated ANGPTL4 levels in the obese subgroup (p=0.0172; t-test). Thus, plasma ANGPTL4 is influenced by body adiposity reflecting ANGPTL4 production by WAT (13-15).

Ectopic (intrahepatic and intramyocellular) lipids measured by magnetic resonance spectroscopy were neither associated with plasma ANGPTL4 in the overall cohort (p≥0.6, all; adjusted for gender, age, and BMI) nor in the lean and obese subgroups (p≥0.07, all; adjusted for gender and age). Thus, circulating ANGPTL4 does not appear to be involved in lipid breakdown in human muscle. This however does not exclude a role of locally produced mANGPTL4 in muscle lipolysis.

As to metabolic traits, plasma ANGPTL4 levels did not reveal significant associations with plasma glucose or insulin concentrations (in the fasting state as well as during OGTT), with OGTT- and HEC-derived indices of insulin sensitivity, or with indices of insulin secretion, such as plasma C-peptide levels at 30min of OGTT and OGTT-derived 1st-phase insulin secretion in the overall cohort (p≥0.24, all; after appropriate adjustments) or in the lean and obese subgroups (p≥0.3, all; after appropriate adjustments). Plasma triglyceride concentrations were also not significantly associated with ANGPTL4 before (p=0.14; adjusted for gender, age, and BMI) and after stratification into lean and obese subjects (p≥0.3, all; adjusted for gender and age).
Hence, the hypertriglyceridemic action of ANGPTL4 detected in mice due to LPL inhibition (16-20) could not be confirmed in this study. However, a very recent genetic study identified a rare mutation in the ANGPTL4 gene, E40K, in European Americans which was associated with lower plasma triglyceride levels clearly pointing to a role of ANGPTL4 in VLDL/chylomicron metabolism in humans (38).

Importantly, plasma ANGPTL4 levels were positively associated with plasma NEFA in the fasting state (Figure 5A) possibly reflecting NEFA-mediated ANGPTL4 production. In addition, plasma ANGPTL4 was positively correlated with plasma NEFA levels during OGTT (Figure 5B), an estimate of WAT lipolysis. Even though these associations cannot constitute causality, these results clearly point to a close relationship between plasma NEFA and ANGPTL4 levels in humans and, in part, confirm earlier findings in mice.

**Relationship between myotube ANGPTL4 expression and metabolic traits of the donors.** The human myotubes used in this study underwent an extended cell culture protocol including isolation from biopsies, expansion, splitting and freezing, storage in liquid nitrogen and thawing, growth to subconfluence, and finally in vitro differentiation. As a result, these cells no longer reflect the metabolic setting in vivo and have lost acquired phenotypes, such as insulin resistance (39). However, some genetically or epigenetically determined features, e.g. susceptibility towards SFA (40) and basal expression of genes (41;42), are maintained and show remarkable inter-individual variation. Therefore, human myotubes represent a model to study the unidirectional effects of genes and their individual expression levels on metabolic parameters of the donors, as discussed earlier (42).

To assess the metabolic role of mANGPTL4 in humans in vivo, we determined basal ANGPTL4 mRNA expression in myotubes from 38 non-diabetic donors (for clinical characteristics, see (41)). From these subjects, plasma glycerol measurements were available in addition to plasma NEFA. In the myotubes, basal ANGPTL4 expression revealed a strong positive correlation with basal PPARD expression (Figure 5C) reflecting the close relationship between PPARδ and its target gene.

By correlational analysis with the donors’ in vivo parameters, the myotube ANGPTL4 mRNA contents were not associated with gender or age (p≥0.5, both). Furthermore, there were no significant correlations with BMI, waist-hip ratio, body fat content, plasma adiponectin and leptin levels, plasma glucose or insulin concentrations (in the fasting state as well as during OGTT), indices of insulin sensitivity or insulin secretion (p≥0.10, all; after appropriate adjustments). Intramyocellular lipid measurements were not available from the donors. Therefore, we have currently no proof of our in vitro finding suggesting involvement of mANGPTL4 in muscle triglyceride breakdown. As to lipid parameters, plasma fasting concentrations of triglycerides, NEFA, and glycerol were not significantly associated with myotube ANGPTL4 expression (p≥0.06, all; adjusted for gender, age, and BMI). However, a significant correlation between myotube ANGPTL4 expression and the area under the curve (AUC) of glycerol during OGTT, an estimate of WAT lipolysis, was detected (Figure 5D). This not only confirms the results obtained with plasma ANGPTL4 measurements, but also indicates that mANGPTL4 is of systemic importance and enhances WAT lipolysis in humans.

To further substantiate the systemic role of mANGPTL4, we additionally measured the plasma ANGPTL4 concentrations of the myotube donors. Importantly, myotube ANGPTL4 expression was significantly
correlated with the donors’ plasma ANGPTL4 levels (Figure 5E). This provides evidence that mANGPTL4 production contributes to circulating ANGPTL4 in humans. The myotube cultures were derived from normal-weight healthy young subjects. Thus, the interesting issue whether myotubes from patients with metabolic disease (obesity, type 2 diabetes) show altered expression/secretion of ANGPTL4 remains to be clarified, and future studies will shed further light on this question.

In summary, we show here that ANGPTL4 is produced and secreted by human myotubes and is subject to gene regulation by major plasma LCFA. Furthermore, we provide evidence for an involvement of PPARδ in LCFA-induced muscle ANGPTL4 expression. These findings could be of physiological relevance in states of increased β-oxidation due to enhanced muscle PPARδ activity and/or PPARD expression, such as fasting (43) and exercise (44-46). In this context, it is conceivable that promotion of WAT lipolysis via PPARδ-mediated mANGPTL4 production represents a mechanism that prevents too strong decrements of plasma NEFA levels and, in this way, ensures ongoing fuel supply of SKM (Figure 6). Such a feed-forward mechanism would favour the efficient use of stored lipids, as opposed to glucose, during periods of increased energy demand. In this scenario, mANGPTL4 would be an important player of crosstalk between SKM and WAT and would explain the loss of WAT mass observed after pharmacological PPARδ activation and/or transgenic PPARD overexpression in mice (44;47;48). Clearly, further studies are needed to confirm this hypothesis. If our preliminary in vitro finding that mANGPTL4 is involved in muscle LIPE expression holds also for the in vivo situation, a synergism of PPARδ actions can be derived in which PPARδ activation, in an ANGPTL4-dependent manner, stimulates breakdown of intramyocellular lipids to fatty acyl-CoA and, in an ANGPTL4-independent manner, promotes acyl-CoA oxidation via induction of β-oxidative enzymes (Figure 6). Finally, this is to our knowledge the first report demonstrating systemic relevance of mANGPTL4 in humans.

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Table 1. Genes induced by both palmitate and linoleate in human myotubes. Cells were treated for 20h with 1.25% BSA for control or 0.5mM LCFA (changes ≥2-fold, LCFA vs. BSA).

| Gene title                                           | Gene symbol | UniGene ID | fold-change by palmitate | fold-change by linoleate |
|------------------------------------------------------|-------------|------------|--------------------------|--------------------------|
| angiopoietin-like 4                                  | ANGPTL4     | Hs.9613    | 50.4                     | 33.5                     |
| granulocyte colony-stimulating factor                | CSF3        | Hs.2233    | 11.3                     | 3.48                     |
| pyruvate dehydrogenase kinase 4                      | PDK4        | Hs.8364    | 8.06                     | 7.89                     |
| biglycan                                             | BGN         | Hs.821     | 6.77                     | 2.45                     |
| adipophillin                                         | ADFP        | Hs.3416    | 5.66                     | 5.98                     |
| signal-induced proliferation-associated protein 1    | SIPA1       | Hs.530477  | 5.39                     | 4.44                     |
| elastin                                              | ELN         | Hs.252418  | 4.34                     | 2.10                     |
| protocadherin γ A12                                  | PCDHGA12    | -          | 3.86                     | 2.27                     |
| spermine oxidase                                     | SMOX        | Hs.433337  | 3.73                     | 3.81                     |
| insulin-like growth factor-binding protein 4          | IGFBP4      | Hs.462998  | 3.51                     | 2.58                     |
| myc-associated zinc finger protein                    | MAZ         | Hs.549052  | 3.48                     | 2.39                     |
| LDL receptor-related protein 1                       | LRP1        | Hs.162757  | 3.47                     | 2.52                     |
| heparan sulfate proteoglycan 2                       | HSPG2       | Hs.550478  | 3.42                     | 2.46                     |
| collagen α3(V) chain                                 | COL5A3      | Hs.235368  | 3.27                     | 2.66                     |
| brain-specific angiogenesis inhibitor 2              | BAI2        | Hs.524138  | 3.25                     | 2.64                     |
| Xaa-Pro aminopeptidase 2                             | XPNPEP2     | Hs.170499  | 3.20                     | 2.33                     |
| transforming growth factor β1                       | TGFBI       | Hs.1103    | 3.16                     | 2.01                     |
| CLIP-170-related 59 kDa protein                      | CLIPR-59    | Hs.466539  | 2.98                     | 2.25                     |
| zinc finger protein 580                              | ZNF580      | Hs.94392   | 2.81                     | 2.00                     |
| vitronectin                                          | VTN         | Hs.2257    | 2.71                     | 2.03                     |
| glucocorticoid receptor DNA-binding factor 1         | GRLF1       | Hs.509447  | 2.64                     | 2.43                     |
| nucleobindin 1                                       | NUCB1       | Hs.515524  | 2.62                     | 2.17                     |
| hyaluronan synthase 1                                | HAS1        | Hs.57697   | 2.62                     | 2.04                     |
| zinc and ring finger 1                               | ZNRF1       | Hs.427284  | 2.60                     | 2.16                     |
| inositol monophosphatase 2                           | IMPA2       | Hs.367992  | 2.57                     | 2.41                     |
| endothelin B receptor                                | EDNRB       | Hs.82002   | 2.51                     | 2.50                     |
| SH3 domain GRB2-like protein B2                      | SH3GLB2     | Hs.460238  | 2.50                     | 2.83                     |
| meteorin, glial cell differentiation regulator-like   | METRN1      | Hs.514615  | 2.45                     | 2.11                     |
| SBF1 protein                                         | SBF1        | Hs.280202  | 2.35                     | 2.13                     |
| glucosidase 2 β-subunit                              | PRKCSH      | Hs.512640  | 2.35                     | 2.04                     |
| mitogen-activated protein kinase 7                   | MAPK7       | Hs.150136  | 2.31                     | 2.06                     |
| replication initiator 1                              | REPIN1      | Hs.521289  | 2.20                     | 2.08                     |
| fos-like antigen 1                                   | FOSL1       | Hs.283565  | 2.14                     | 2.06                     |
| 5'-TG-3'-interacting factor                          | TGF1        | Hs.373550  | 2.07                     | 2.16                     |
| KIAA0467 protein                                     | KIAA0467    | Hs.301943  | 2.03                     | 2.19                     |
Figure 1. ANGPTL4 production by human myotubes and its regulation by LCFA

Cells were treated for 20h with 1.25% BSA (control for myristate, palmitate, palmitoleate, oleate, and linoleate), 2.5% BSA (control for stearate and palmitate+linoleate), or 0.5mM of each LCFA. A and C) Induction of the PPAR target genes ANGPTL4 (A) and PDK4 (C) by LCFA. RNA was quantified by real-time RT-PCR (RAU – relative arbitrary units). B) Intracellular ANGPTL4 protein levels. ANGPTL4 protein was measured by ELISA and normalized to cellular protein contents. Statistics: p=0.0008 (A), p=0.0133 (B), and p<0.0001 (C); ANOVA; n≥4; *significantly different from 1.25% BSA (post hoc p<0.05); **significantly different from 2.5% BSA (post hoc p<0.05).
Figure 2. Induction of ANGPTL4 production in human myotubes by pharmacological PPARδ activation

A) Induction of ANGPTL4 expression by isoform-specific PPAR agonists. Cells were treated for 20h with 0.1% DMSO (carrier control), 10µM Wy-14,643, 60µM fenofibrate, 10µM troglitazone, 1µM rosiglitazone, or 1µM GW501516. RNA was quantified by real-time RT-PCR. Statistics: p<0.0001; ANOVA; n≥4; *significantly different from DMSO (post hoc p<0.05). B and C) Time-dependent induction of ANGPTL4 (B) and PDK4 (C) by GW501516. Cells were treated for 48h with 0.1% DMSO or 1µM GW501516. RNA was quantified by real-time RT-PCR. Statistics: *significant differences between treatment groups over time: p<0.0001 (B) and p=0.0021 (C); time vs. treatment; MANOVA; n=3. D) Time-dependent stimulation of ANGPTL4 secretion by GW501516. Cells were treated for 48h with 0.1% DMSO or 1µM GW501516. ANGPTL4 secreted into the culture supernatant was quantified by ELISA. Statistics: *significant differences between treatment groups over time: p<0.0001; time vs. treatment; MANOVA; n=3. E) Cleavage of secreted ANGPTL4 during GW501516 treatment. Cells were treated for 48h with 1µM GW501516. Supernatants conditioned by human myotube cultures derived from two donors were subjected to immunoblot analysis.
Figure 3. Role of PPARδ in LCFA-induced ANGPTL4 expression in C2C12 myocytes
A) Regulation of C2C12 ANGPTL4 expression by LCFA. Cells were treated for 20h with 1.25% BSA, 2.5% BSA, or 0.5mM of each LCFA. RNA was quantified by real-time RT-PCR (RAU – relative arbitrary units). Statistics: p=0.0012; ANOVA; n=3; *significantly different from 1.25% BSA (post hoc p<0.05); **significantly different from 2.5% BSA (post hoc p<0.05). B) Time-dependent induction of ANGPTL4 by GW501516. Cells were treated for 20h with 0.1% DMSO or 1µM GW501516. RNA was quantified by real-time RT-PCR. Statistics: *significant differences between treatment groups over time: p<0.0001; time vs. treatment; MANOVA; n=3. C) Knock-down of C2C12 PPARδ expression by RNAi. Cells were left untreated (basal) or were treated for 8h with siRNA directed against bacterial luciferase (for control) or PPARδ, respectively. Cells were lysed after siRNA wash-out and incubation with fresh medium for 16h. RNA was quantified by real-time RT-PCR. Statistics: p<0.0001; ANOVA; n=3; *significantly different from basal (post hoc p<0.05). D) Oleate-induced ANGPTL4 expression of C2C12 cells after PPARδ knock-down. Cells were left untreated or were treated for 8h with siRNA directed against bacterial luciferase (for control) or PPARδ, respectively. After siRNA wash-out and 16-h incubation with fresh medium, cells were treated for 20h with 1.25% BSA or 0.5mM oleate. RNA was quantified by real-time RT-PCR. Statistics: p=0.0217; ANOVA; n=3; *significantly different from BSA (post hoc p<0.05); **significantly different from oleate + control siRNA (post hoc p<0.05). E) Oleate-induced recruitment of PPARδ to the ANGPTL4 gene. Cells were treated for 6h with 1.25% BSA or 0.5mM oleate, respectively. After cross-linking with formaldehyde, cells were subjected to anti-PPARδ ChIP. The co-immunoprecipitated DNA was analysed for the presence of ANPTL4 DNA using PCR amplification of a 310-bp fragment harbouring the PPRE in intron 3.
Figure 4. ANGPTL4-dependent LIPE expression in C2C12 myocytes
A and B) Time-dependent induction of C2C12 LIPE (A) and PNPLA2 (B) by GW501516. Cells were treated for 48h with 0.1% DMSO or 1µM GW501516. RNA was quantified by real-time RT-PCR (RAU – relative arbitrary units). Statistics: *significant differences between treatment groups over time: p=0.0007 (A) and p=0.0002 (B); time vs. treatment; MANOVA; n=3. C) Knock-down of C2C12 ANGPTL4 expression by RNAi. Cells were treated for 8h with 1µM GW501516 alone (control) or with GW501516 in combination with siRNA directed against bacterial luciferase or ANGPTL4, respectively. Cells were lysed after siRNA wash-out and incubation with fresh GW501516-containing medium for 16h. RNA was quantified by real-time RT-PCR. Statistics: p<0.0001; ANOVA; n=3; *significantly different from control (post hoc p<0.05). D) C2C12 LIPE expression after ANGPTL4 knock-down. Cells were treated as described above (see C). RNA was quantified by real-time RT-PCR. Statistics: p=0.0063; ANOVA; n=3; *significantly different from DMSO (post hoc p<0.05); **significantly different from GW501516 + control siRNA (post hoc p<0.05).
Figure 5. Association of ANGPTL4 with lipid metabolism in humans

A and B) Association of human plasma ANGPTL4 with fasting NEFA (A) and WAT lipolysis (B). Plasma ANGPTL4 and NEFA were determined in 108 subjects. (A) Fasting plasma NEFA were adjusted for gender, age, and BMI. (B) As an estimate of WAT lipolysis, the AUC of NEFA during OGTT was used and adjusted for gender, age, BMI, and the AUC of insulin during OGTT. Adjustments were achieved by multivariate linear regression modelling. C, D, and E) Association of basal human myotube ANGPTL4 expression with basal PPARD expression (C), WAT lipolysis of the donors (D), and plasma ANGPTL4 levels of the donors (E). RNA was quantified by real-time RT-PCR (RAU – relative arbitrary units). Glycerol and ANGPTL4 levels were measured in plasma. Data derived from 38 human donors are plotted. (C and E) Unadjusted data are shown. (D) As an estimate of WAT lipolysis, the AUC of glycerol during OGTT was used and adjusted for gender, age, BMI, and the AUC of insulin during OGTT. Adjustments were achieved by multivariate linear regression modelling.
Figure 6. Hypothetical model of mANGPTL4’s role in lipid metabolism
In states of increased muscle PPARδ activity and/or PPARD expression, such as fasting and exercise, SKM produces and secretes ANGPTL4. Simultaneously, muscular fatty acid oxidation is increased by PPARδ-dependent induction of β-oxidative enzymes. Via the circulation, mANGPTL4 enhances WAT lipolysis and thus prevents too strong decrements of plasma NEFA levels and ensures ongoing fuel supply of the stressed (fasting or working) muscle. Together with ANGPTL4’s inhibitory effect on LPL, this mechanism is expected to provoke loss of WAT mass. Furthermore, as derived from our in vitro data, ANGPTL4 could stimulate SKM lipolysis via LIPE induction in an auto-/paracrine manner. This effect would constitute, in addition to PPARδ’s inductive effect on β-oxidation, a synergistic mode of PPARδ action on muscle lipid catabolism (IMCL – intramyocellular lipids).