Muscle-Derived Angiopoietin-Like Protein 4 Is Induced by Fatty Acids via Peroxisome Proliferator–Activated Receptor (PPAR)-δ and Is of Metabolic Relevance in Humans

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OBJECTIVE—Long-chain fatty acids (LCFAs) 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 PPAR-α or -γ, and pharmacological activation of PPAR-δ markedly induced ANGPTL4 production and secretion. In C2C12 myocytes, knockdown of *PPARD*, but not of *PPARG*, blocked LCFA-mediated *ANGPTL4* induction, and LCFA treatment resulted in PPAR-δ recruitment to the *ANGPTL4* gene. In addition, pharmacological PPAR-δ activation induced *LIPE* (encoding hormone-sensitive lipase), and this response crucially depended on ANGPTL4, as revealed by *ANGPTL4* knockdown. In a human cohort of 108 thoroughly phenotyped subjects, plasma ANGPTL4 positively correlated with fasting nonesterified 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 PPAR-δ, and muscle-derived ANGPTL4 seems to be of systemic relevance in humans. ***Diabetes* 58: 579–589, 2009**

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The metabolic syndrome, 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 nonesterified fatty acid (NEFA) levels are frequently observed in metabolic syndrome 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 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 saturated fatty acids exert direct gene regulatory effects and may also in this way contribute to metabolic syndrome (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, saturated fatty acids 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, such as palmitoleate, oleate, and linoleate, increase mitochondrial activity of SKM cells by induction of PPAR-γ coactivator-1α (10).

Even though LCFA-regulated transcription factors (NF-κB, PPARs) are known to date, LCFA-dependent gene

TABLE 1
Genes induced by both palmitate and linoleate in human myotubes

	Gene symbol	UniGene ID	Fold-change by palmitate	Fold-change by linoleate
Angiopoietin-like 4	ANGPTL4	Hs0.9613	50.4	33.5
Granulocyte colony-stimulating factor	CSF3	Hs0.2233	11.3	3.48
Pyruvate dehydrogenase kinase 4	PDK4	Hs0.8364	8.06	7.89
Biglycan	BGN	Hs0.821	6.77	2.45
Adipophilin	ADFP	Hs0.3416	5.66	5.98
Signal-induced proliferation-associated protein 1	SIPA1	Hs0.530477	5.39	4.44
Elastin	ELN	Hs0.252418	4.34	2.10
Protocadherin γ A12	PCDHGA12	-	3.86	2.27
Spermine oxidase	SMOX	Hs0.433337	3.73	3.81
Insulin-like growth factor-binding protein 4	IGFBP4	Hs0.462998	3.51	2.58
myc-associated zinc finger protein	MAZ	Hs0.549052	3.48	2.39
LDL receptor-related protein 1	LRP1	Hs0.162757	3.47	2.52
Heparan sulfate proteoglycan 2	HSPG2	Hs0.550478	3.42	2.46
Collagen α3(V) chain	COL5A3	Hs0.235368	3.27	2.66
Brain-specific angiogenesis inhibitor 2	BAI2	Hs0.524138	3.25	2.64
Xaa-Pro aminopeptidase 2	XPNPEP2	Hs0.170499	3.20	2.33
Transforming growth factor β1	TGFB1	Hs0.1103	3.16	2.01
CLIP-170-related 59 kDa protein	CLIPR-59	Hs0.466539	2.98	2.25
Zinc finger protein 580	ZNF580	Hs0.94392	2.81	2.00
Vitronectin	VTN	Hs0.2257	2.71	2.03
Glucocorticoid receptor DNA-binding factor 1	GRLF1	Hs0.509447	2.64	2.43
Nucleobindin 1	NUCB1	Hs0.515524	2.62	2.17
Hyaluronan synthase 1	HAS1	Hs0.57697	2.62	2.04
Zinc and ring finger 1	ZNRF1	Hs0.427284	2.60	2.16
Inositol monophosphatase 2	IMPA2	Hs0.367992	2.57	2.41
Endothelin B receptor	EDNRB	Hs0.82002	2.51	2.50
SH3 domain GRB2-like protein B2	SH3GLB2	Hs0.460238	2.50	2.83
Meteorin, glial cell differentiation regulator-like	METRNL	Hs0.514615	2.45	2.11
SBF1 protein	SBF1	Hs0.280202	2.35	2.13
Glucosidase 2 β-subunit	PRKCSH	Hs0.512640	2.35	2.04
Mitogen-activated protein kinase 7	MAPK7	Hs0.150136	2.31	2.06
Replication initiator 1	REPIN1	Hs0.521289	2.20	2.08
fos-like antigen 1	FOSL1	Hs0.283565	2.14	2.06
5′-TG-3′ interacting factor	TGIF	Hs0.373550	2.07	2.16
KIAA0467 protein	KIAA0467	Hs0.301943	2.03	2.19

Cells were treated for 20 h with 1.25% BSA for control or 0.5 mmol/l LCFA (changes ≥2-fold, LCFA vs. BSA).

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 the metabolic syndrome.

RESEARCH DESIGN AND METHODS

A detailed description of the methods is given in the online appendix (found at http://diabetes.diabetesjournals.org/cgi/content/full/db07-1438/DC1).

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 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–PPAR-δ antibody K-20 from Santa Cruz Biotechnology.

All 38 myotube donors underwent an oral glucose tolerance test (OGTT) and a hyperinsulinemic-euglycemic clamp. The 108 subjects with plasma ANGPTL4 measurements were characterized by OGTT and a subgroup of 91 subjects also by hyperinsulinemic-euglycemic clamp. All subjects gave informed written consent to the study. The protocol was approved by the local ethics committee. Total, visceral, and nonvisceral fat was determined by magnetic resonance imaging. Intramyocellular and intrahepatic lipids were measured by magnetic resonance spectroscopy. Glucose, insulin, C-peptide, NEFA, glycerol, triglycerides, and ANGPTL4 were quantified by standard laboratory methods.

RESULTS AND DISCUSSION

Gene array analysis. In an initial attempt to identify novel LCFA target genes related to metabolic syndrome, we treated human myotubes derived from a healthy male German donor with bovine serum albumin (BSA; carrier control), the saturated fatty acid palmitate, or the unsaturated fatty acid linoleate and performed whole-genome gene array analysis. Palmitate treatment repressed 181 and induced 316 genes, and linoleate repressed 30 and induced 104 genes. Thus, LCFAs appear to influence the expression of numerous genes in human myotubes. Notably, only five genes were repressed (Supplementary Table 3, found in the online appendix), 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 nonreplicated 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 PPAR-γ 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 knockout, and 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 is 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, *ANPTL4* expression was consistently found upregulated 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 ~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 LCFAs 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- to 50-fold by all LCFAs tested (Fig. 1*A*). The *ANGPTL4* mRNA levels found after treatment with palmitate, stearate, palmitoleate, oleate, linoleate, or a combination of palmitate and linoleate were significantly different from their respective BSA controls (Fig. 1*A*). 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 depended neither 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 LCFAs increased intracellular ANGPTL4 protein 1.5- to 2.3-fold, with palmitate, oleate, and linoleate reaching statistical significance (Fig. 1*B*). 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

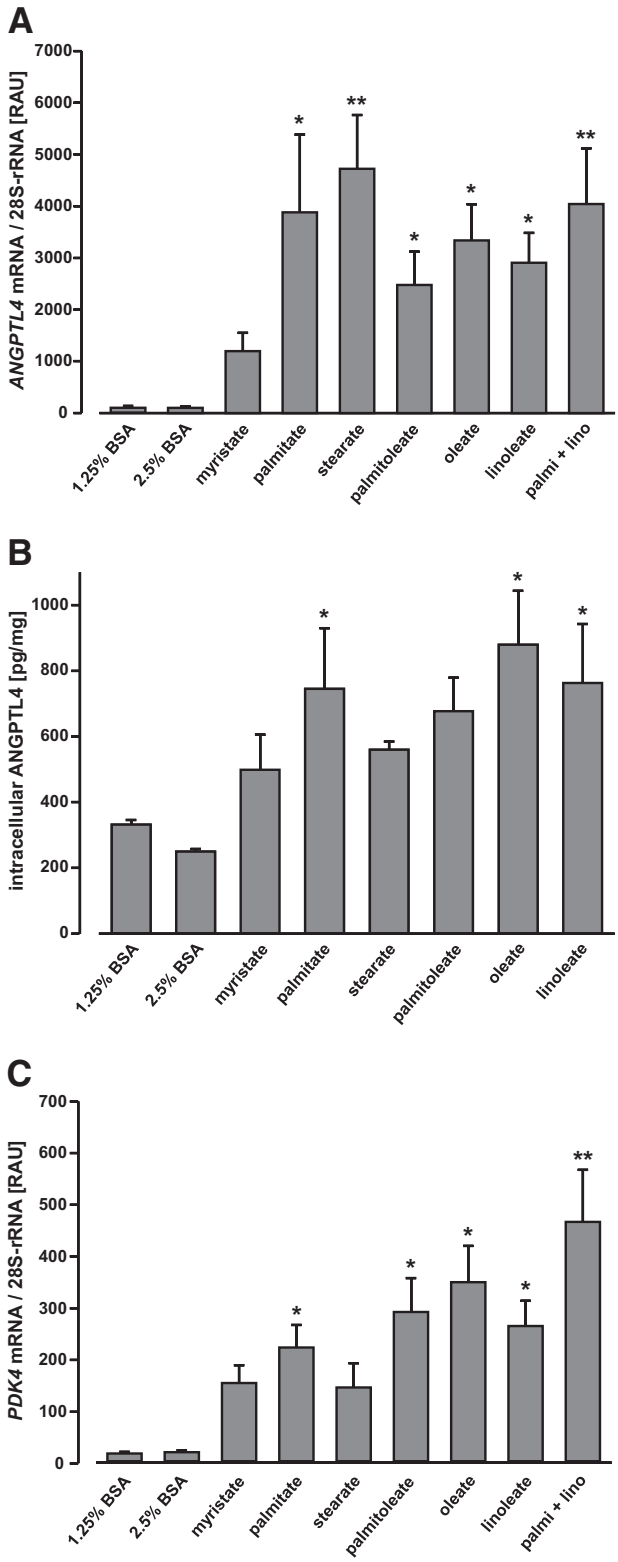

FIG. 1. ANGPTL4 production by human myotubes and its regulation by LCFAs. Cells were treated for 20 h with 1.25% BSA (control for myristate, palmitate, palmitoleate, oleate, and linoleate), 2.5% BSA (control for stearate and palmitate + linoleate), or 0.5 mmol/l of each LCFA. *A* and *C*: Induction of the PPAR target genes *ANGPTL4* (*A*) and *PDK4* (*C*) by LCFA. RNA was quantified by real-time RT-PCR (relative arbitrary units [RAU]). *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; $n \geq 4$; *significantly different from 1.25% BSA (post hoc $P < 0.05$); **significantly different from 2.5% BSA (post hoc $P < 0.05$).

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 25 mmol/l glucose (added to glucose-free medium). After 20 h, there was no significant glucose effect on basal myotube *ANGPTL4* expression ($P = 0.9$, ANOVA; $n = 4$).

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

Modulation of human myotube PPAR isoform expression and activation by LCFAs. *ANGPTL4* is a known target gene of PPAR-α and -δ in liver (14,22) and PPAR-γ in WAT (14,15), and a PPAR response element was identified in intron 3 of the *ANGPTL4* gene (23). To see whether LCFAs enhance myotube *ANGPTL4* expression via induction of the genes encoding PPAR-α, -γ, or -δ (*PPARA*, *PPARG*, or *PPARD*, respectively), we quantified the respective mRNA levels before and after LCFA treatment. In untreated human myotubes, the *PPARD* mRNA contents were ~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 with their respective BSA controls ($P \geq 0.2$, both, ANOVA; $n = 5$). Furthermore, all LCFAs shown above to induce *ANGPTL4* (Fig. 1*A*) 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 LCFAs tested, including stearate, provoked relevant changes in PPAR-δ protein after 20 h of treatment ($n = 3$; data not shown). Therefore, we suggest that LCFAs act as PPAR ligands or activators (intracellularly metabolized to ligands), but not as regulators of PPAR expression/production.

To examine whether LCFAs 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 a target of PPAR-δ and -α (24), *CD36* (encoding fatty acid translocase) and *UCP3* (encoding uncoupling protein 3) as PPAR-α–specific targets (25,26), and *PPARG* as a target of PPAR-γ itself (27). Compared with their respective BSA controls, none of the LCFAs modulated the expression of *CD36*, *UCP3*, or *PPARG* in these cells ($P \geq 0.4$, all, ANOVA; $n \geq 4$). By contrast, all LCFA induced *PDK4* mRNA expression 7- to 22-fold, and the effects of palmitate, palmitoleate, oleate, linoleate, and the combination of palmitate and linoleate were statistically significant (Fig. 1*C*). This led us to assume that the LCFAs tested in this study selectively activate PPAR-δ in human myotubes, at least at the concentration tested (0.5 mmol/l).

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 PPAR-α–specific fibrates Wy-14,643 and fenofibrate, the PPAR-γ–specific thiazolidinediones troglitazone and rosiglitazone, and the PPAR-δ–specific activator GW501516. As presented in Fig. 2*A*, *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 10-fold higher affinity for PPAR-γ than troglitazone, nor by any of the fibrates tested. Only GW501516 treatment reached a level of *ANGPTL4* induction comparable to that found with LCFAs. Furthermore, *ANGPTL4* induction by GW501516 followed the same kinetics as the other PPAR-δ target gene *PDK4* (Figs. 2*B* and 2*C*). We also tested GW501516's effect on intracellular ANGPTL4 protein contents using ELISA. GW501516 treatment (1 μmol/l, 20 h) increased intracellular ANGPTL4 protein 2.5-fold over DMSO control (365.6 ± 52.9 vs. 908.2 ± 154.0 pg/mg; $P = 0.0158$, t test; $n = 4$). These data strengthened our suggestion of PPAR-δ 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 (Fig. 2*D*). 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 (~70 kDa), a 50-kDa COOH-terminal and a 26-kDa NH_2-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 COOH-terminal fragment accumulated in the culture supernatant during the treatment period (Fig. 2*E*). The NH_2-terminal fragment could not be detected with antibodies from different suppliers directed against full-length or the NH_2-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 PPAR-δ in LCFA-enhanced *ANGPTL4* expression in C2C12 myocytes. To evidence that LCFA-induced myocyte *ANGPTL4* expression depends on PPAR-δ, 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 saturated fatty acid and unsaturated fatty acid species. As presented in Fig. 3*A*, all LCFAs tested increased the C2C12 *ANGPTL4* mRNA contents when compared with 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 (Fig. 3*B*). As depicted in Fig. 3*C*, transfection with siRNA directed against *PPARD*, but not with control siRNA directed against bacterial luciferase, reduced C2C12 *PPARD* mRNA contents by 82%. After *PPARD* knockdown, oleate-induced *ANGPTL4* expression was significantly impaired (2.4-fold in the presence vs. 6.8-fold in the absence of *PPARD* siRNA); moreover, the oleate effect was no longer significant (Fig. 3*D*). Furthermore, control transfection with siRNA directed against bacterial luciferase still allowed marked *ANGPTL4* induction by oleate

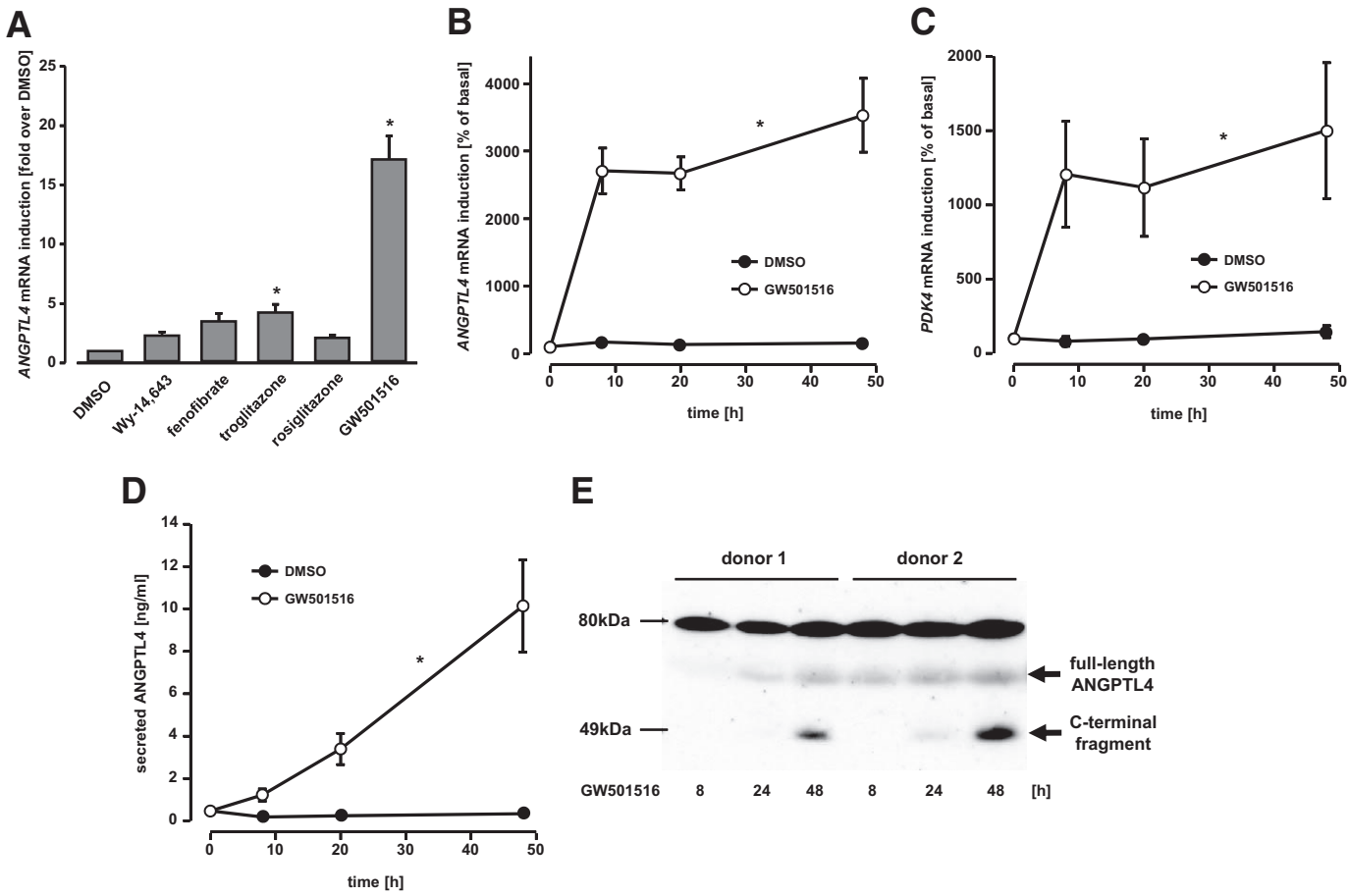

FIG. 2. Induction of ANGPTL4 production in human myotubes by pharmacological PPAR-δ activation. *A*: Induction of *ANGPTL4* expression by isoform-specific PPAR agonists. Cells were treated for 20 h with 0.1% DMSO (carrier control), 10 μmol/l Wy-14,643, 60 μmol/l fenofibrate, 10 μmol/l troglitazone, 1 μmol/l rosiglitazone, or 1 μmol/l GW501516. RNA was quantified by real-time RT-PCR. Statistics: $P < 0.0001$; ANOVA; $n \geq 4$; *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 48 h with 0.1% DMSO or 1 μmol/l 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 versus treatment; MANOVA; $n = 3$. *D*: Time-dependent stimulation of ANGPTL4 secretion by GW501516. Cells were treated for 48 h with 0.1% DMSO or 1 μmol/l GW501516. ANGPTL4 secreted into the culture supernatant was quantified by ELISA. Statistics: *significant differences between treatment groups over time: $P < 0.0001$; time versus treatment; MANOVA; $n = 3$. *E*: Cleavage of secreted ANGPTL4 during GW501516 treatment. Cells were treated for 48 h with 1 μmol/l GW501516. Supernatants conditioned by human myotube cultures derived from two donors were subjected to immunoblot analysis.

(Fig. 3*D*). Thus, induction of *ANGPTL4* expression in C2C12 myocytes by the representative LCFA oleate requires PPAR-δ. 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 PPAR-δ.

To ultimately prove that PPAR-δ is activated by LCFAs and binds to the *ANGPTL4* gene, we performed ChIP analysis in C2C12 cells treated for 6 h with oleate. In the anti–PPAR-δ immunoprecipitate, markedly more *ANGPTL4* intron 2 DNA (harboring the PPRE) could be detected in oleate-treated cells compared with BSA-treated cells (Fig. 3*E*). This demonstrates that PPAR-δ 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 (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 adipose triglyceride lipase) and 11.8 ± 2.0 fg *LIPE* mRNA (encoding hormone-sensitive lipase)/μg total RNA (means ± SD; $n = 3$). Treatment of C2C12 cells with GW501516 provoked significant increments over time of *LIPE* and *PNPLA2* mRNA (Fig. 4*A* and *B*) compared with DMSO (carrier control). However, the kinetics of both gene regulations was completely different from that of the aforementioned PPAR-δ target genes *PDK4* and *ANGPTL4* in that these lipases showed delayed induction (compare Fig. 2*B* and C with Fig. 4*A* and *B*). This suggests that *LIPE* and *PNPLA2* are not direct PPAR-δ target genes.

The GW501516 effect on *PNPLA2* expression was only obvious at very late time points (beyond 24 h after start of treatment) when DMSO alone also revealed some

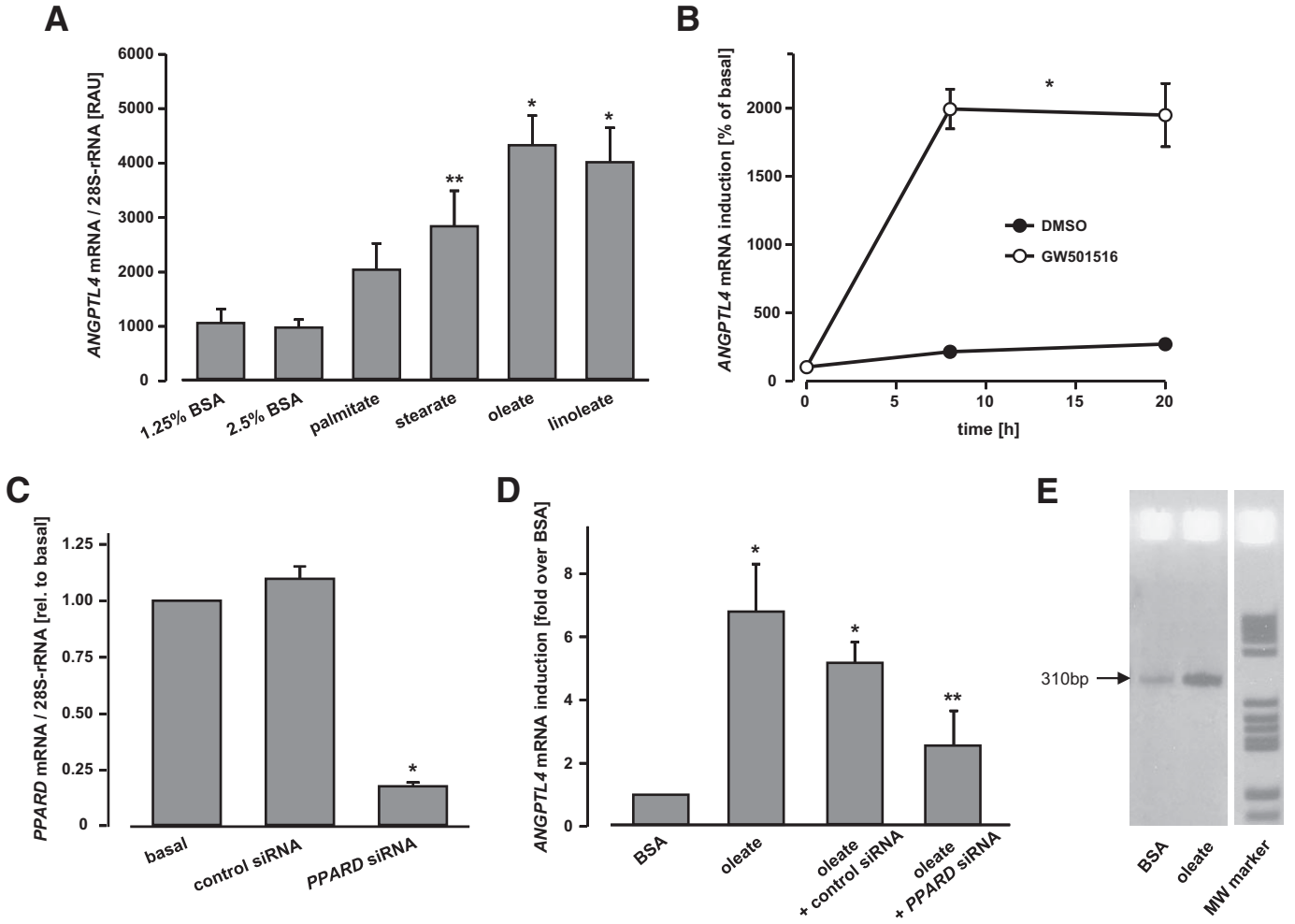

FIG. 3. Role of PPAR-δ in LCFA-induced *ANGPTL4* expression in C2C12 myocytes. *A*: Regulation of C2C12 *ANGPTL4* expression by LCFAs. Cells were treated for 20 h with 1.25% BSA, 2.5% BSA, or 0.5 mmol/l of each LCFA. RNA was quantified by real-time RT-PCR (relative arbitrary units [RAU]). Statistics: $P = 0.0012$; 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 20 h with 0.1% DMSO or 1 μmol/l GW501516. RNA was quantified by real-time RT-PCR. Statistics: *significant differences between treatment groups over time: $P < 0.0001$; time versus treatment; MANOVA; $n = 3$. *C*: Knockdown of C2C12 *PPARD* expression by RNAi. Cells were left untreated (basal) or were treated for 8 h with siRNA directed against bacterial luciferase (for control) or *PPARD*, respectively. Cells were lysed after siRNA washout and incubation with fresh medium for 16 h. 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 *PPARD* knockdown. Cells were left untreated or were treated for 8 h with siRNA directed against bacterial luciferase (for control) or *PPARD*, respectively. After siRNA washout and 16-h incubation with fresh medium, cells were treated for 20 h with 1.25% BSA or 0.5 mmol/l 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 PPAR-δ to the *ANGPTL4* gene. Cells were treated for 6 h with 1.25% BSA or 0.5 mmol/l oleate, respectively. After cross-linking with formaldehyde, cells were subjected to anti–PPAR-δ ChIP. The co-immunoprecipitated DNA was analyzed for the presence of *ANPTL4* DNA using PCR amplification of a 310-bp fragment harboring the PPRE in intron 3.

gene regulatory effects. Using RNAi, we therefore assessed here the potential autocrine/paracrine role of myocyte *ANGPTL4* expression during the moderately delayed stage of *LIPE* expression (at 20 h of treatment). As depicted in Fig. 4*C*, 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* knockdown significantly impaired GW501516-mediated *LIPE* induction (1.6-fold in the presence vs. 2.6-fold in the absence of *ANGPTL4* siRNA); in addition, the GW501516 effect was no longer significant (Fig. 4*D*). Again, control transfection with bacterial luciferase siRNA did not significantly alter the GW501516 effect on *LIPE* expression (Fig. 4*D*).

Here, we show that pharmacological PPAR-δ activation upregulates *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 PPAR-δ agonists (32). Moreover, this finding could point to an autocrine/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 PPAR-δ gain- and loss-of-function animal models, are clearly needed. Because 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. Because 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

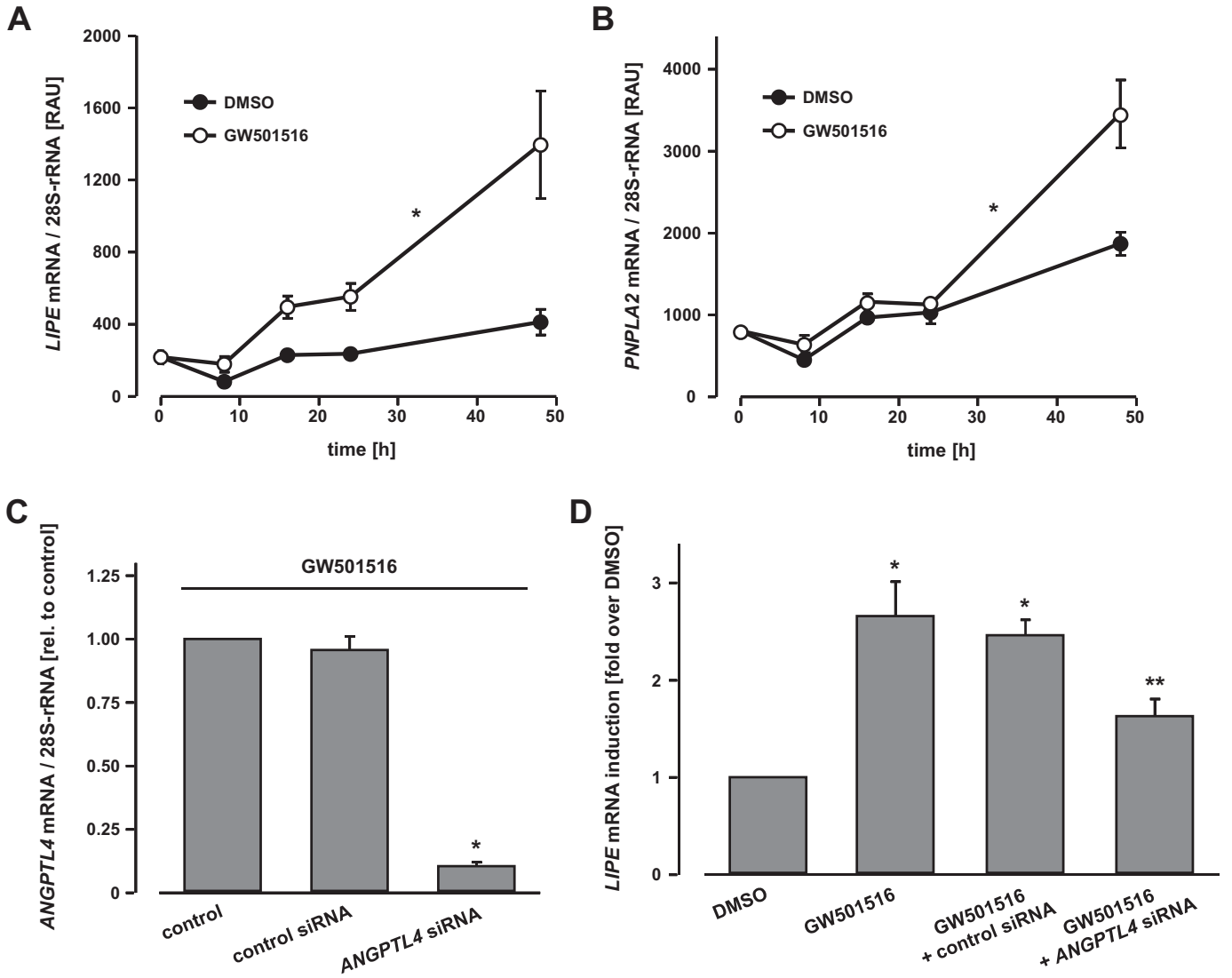

FIG. 4. ANGPTL4-dependent *LIPE* expression in C2C12 myocytes. *A* and *B*: Time-dependent induction of C2C12 *LIPE* (*A*) and *PNPLA2* (*B*) by GW501516. Cells were treated for 48 h with 0.1% DMSO or 1 μmol/l GW501516. RNA was quantified by real-time RT-PCR (relative arbitrary units [RAU]). Statistics: *significant differences between treatment groups over time: $P = 0.0007$ (*A*) and $P = 0.0002$ (*B*); time versus treatment; MANOVA; $n = 3$. *C*: Knockdown of C2C12 *ANGPTL4* expression by RNAi. Cells were treated for 8 h with 1 μmol/l GW501516 alone (control) or with GW501516 in combination with siRNA directed against bacterial luciferase or *ANGPTL4*, respectively. Cells were lysed after siRNA washout and incubation with fresh GW501516-containing medium for 16 h. 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* knockdown. 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 $P < 0.05$).

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 are presented in Supplementary Table 2) using ELISA.

The mean plasma ANGPTL4 concentration measured was 1.73 ± 0.11 ng/ml (means ± SE; range 0.37–8.00 ng/ml). The plasma ANGPTL4 levels were not correlated with sex 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-to-hip ratio; body fat measured by bioelectrical impedance; total, visceral, and nonvisceral fat mass measured by magnetic resonance imaging; or plasma levels of adiponectin and leptin ($P \geq 0.06$, all, after appropriate adjustments). However, stratification of the cohort into lean (BMI <27 kg/m^2, 19.5–26.9 kg/m^2; $n = 30$) and obese (BMI ≥30 kg/m^2, 30.0–48.4 kg/m^2; $n = 39$) 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 ($P \geq 0.6$, all; adjusted for sex, age, and BMI) nor in the lean and obese subgroups ($P \geq 0.07$, all; adjusted for sex 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 hyperinsulinemic-euglycemic clamp–derived indexes of insulin sensitivity, or with

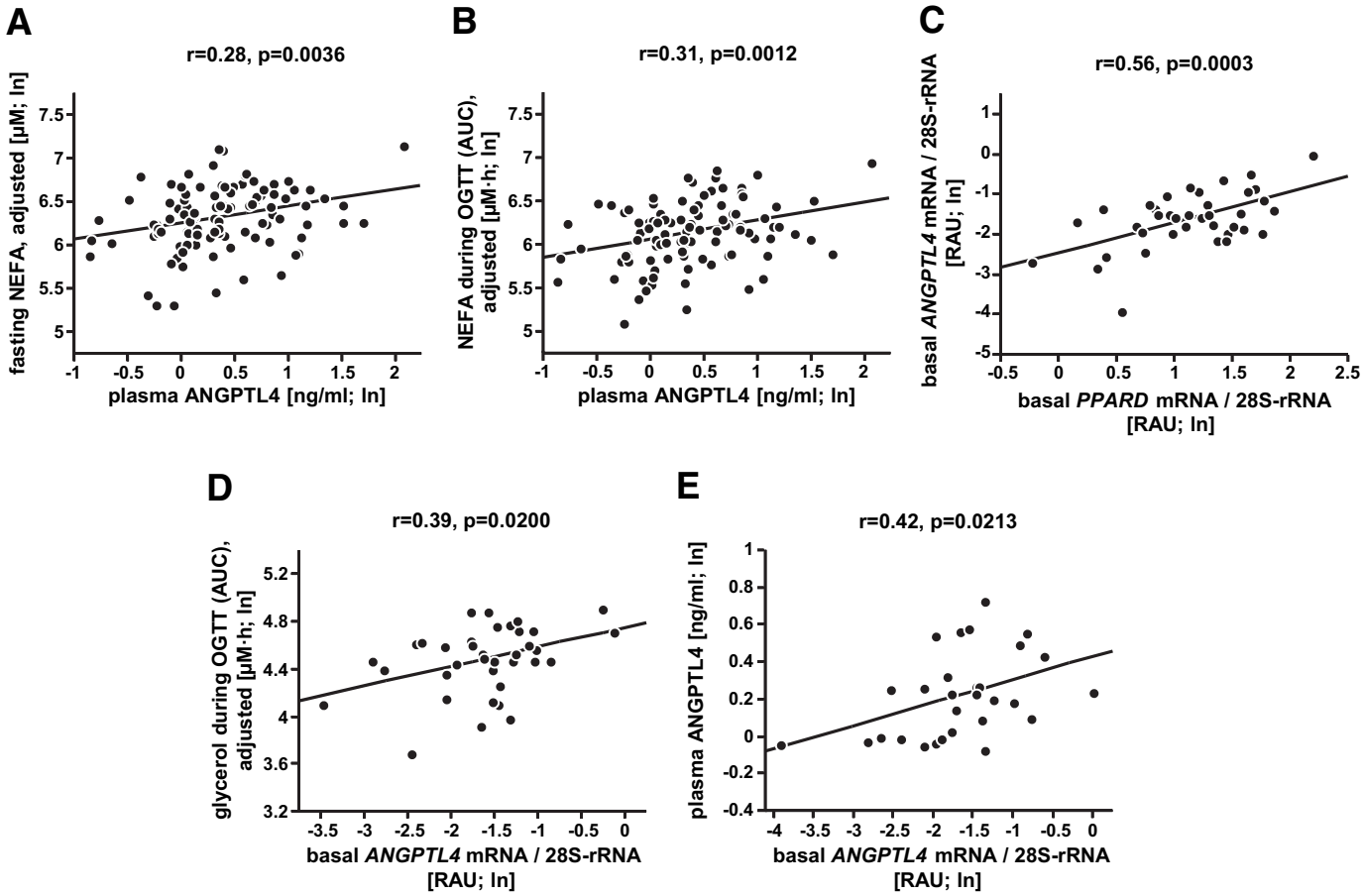

FIG. 5. Association of ANGPTL4 with lipid metabolism in humans. *A* and *B*: Association of human plasma ANGPTL4 with fasting NEFA (*A*) and WAT lipolysis (*B*). Plasma ANGPTL4 and NEFA were determined in 108 subjects. *A*: Fasting plasma NEFAs were adjusted for sex, age, and BMI. *B*: As an estimate of WAT lipolysis, the area under the curve (AUC) of NEFA during OGTT was used and adjusted for sex, age, BMI, and the AUC of insulin during OGTT. Adjustments were achieved by multivariate linear regression modeling. *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 (relative arbitrary units [RAU]). Glycerol and ANGPTL4 levels were measured in plasma. Data derived from 38 human donors are plotted. *C* and *E*: Unadjusted data are shown. In *D*, as an estimate of WAT lipolysis, the AUC of glycerol during OGTT was used and adjusted for sex, age, BMI, and the AUC of insulin during OGTT. Adjustments were achieved by multivariate linear regression modeling.

indexes of insulin secretion, such as plasma C-peptide levels at 30 min of OGTT and OGTT-derived first-phase insulin secretion in the overall cohort ($P \geq 0.24$, all, after appropriate adjustments) or in the lean and obese subgroups ($P \geq 0.3$, all, after appropriate adjustments). Plasma triglyceride concentrations were also not significantly associated with ANGPTL4 before ($P = 0.14$; adjusted for sex, age, and BMI) and after stratification into lean and obese subjects ($P \geq 0.3$, all; adjusted for sex 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 NEFAs in the fasting state (Fig. 5*A*), possibly reflecting NEFA-mediated ANGPTL4 production. In addition, plasma ANGPTL4 was positively correlated with plasma NEFA levels during OGTT (Fig. 5*B*), 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 toward saturated fatty acids (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 nondiabetic donors (for clinical characteristics, see Staiger et al. [41]). From these subjects, plasma glycerol measurements were available in addition to plasma NEFAs. In the myotubes, basal *ANGPTL4* expression revealed a strong positive correla-

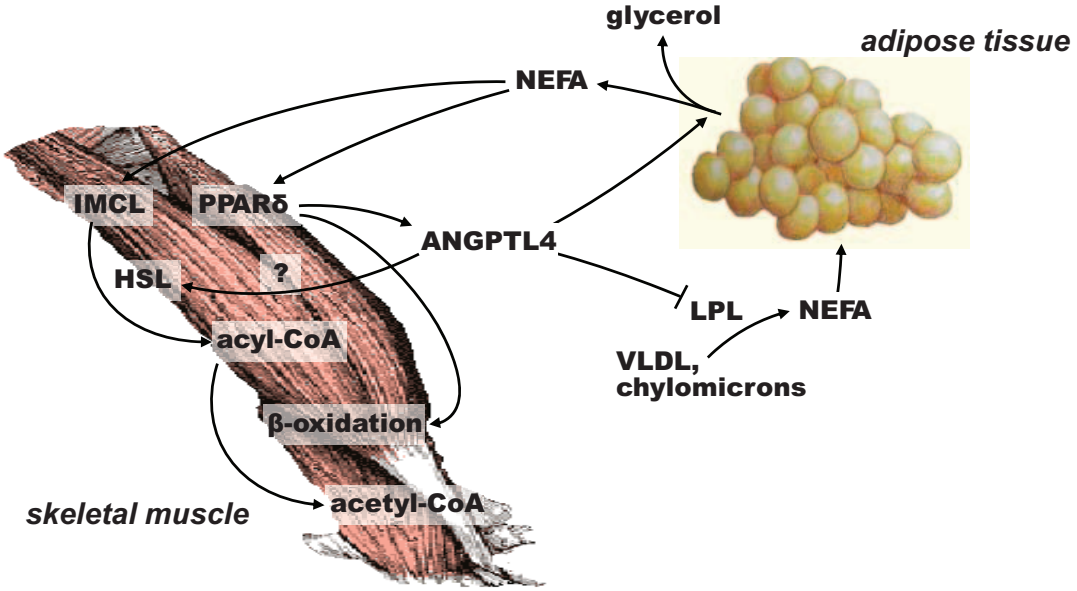

FIG. 6. Hypothetical model of mANGPTL4's role in lipid metabolism. In states of increased muscle PPAR-δ activity and/or *PPARD* expression, such as fasting and exercise, SKM produces and secretes ANGPTL4. Simultaneously, muscular fatty acid oxidation is increased by PPAR-δ–dependent induction of β-oxidative 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 autocrine/paracrine manner. This effect would constitute, in addition to PPAR-δ's inductive effect on β-oxidation, a synergistic mode of PPAR-δ action on muscle lipid catabolism (IMCL, intramyocellular lipids).

tion with basal *PPARD* expression (Fig. 5*C*) reflecting the close relationship between PPAR-δ and its target gene.

By correlational analysis with the donors' in vivo parameters, the myotube *ANGPTL4* mRNA contents were not associated with sex or age ($P \geq 0.5$, both). Furthermore, there were no significant correlations with BMI, waist-to-hip ratio, body fat content, plasma adiponectin and leptin levels, plasma glucose or insulin concentrations (in the fasting state as well as during OGTT), or indexes of insulin sensitivity or insulin secretion ($P \geq 0.10$, 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 ($P \geq 0.06$, all; adjusted for sex, age, and BMI). However, a significant correlation between myotube *ANGPTL4* expression and the area under the curve of glycerol during OGTT, an estimate of WAT lipolysis, was detected (Fig. 5*D*). 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 (Fig. 5*E*). 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 of 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 LCFAs. Furthermore, we provide evidence for an involvement of PPAR-δ in LCFA-induced muscle *ANGPTL4* expression. These findings could be of physiological relevance in states of increased β-oxidation due to enhanced muscle PPAR-δ 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 PPAR-δ–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 (Fig. 6). Such a feed-forward mechanism would favor 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 PPAR-δ 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 PPAR-δ actions can be derived in which PPAR-δ 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 (Fig. 6). Finally, this is to our knowledge the first report demonstrating systemic relevance of mANGPTL4 in humans.

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No potential conflicts of interest relevant to this article were reported.

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