

The Fasting-induced Adipose Factor/Angiopoietin-like Protein 4 Is Physically Associated with Lipoproteins and Governs Plasma Lipid Levels and Adiposity*

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Proteins secreted from adipose tissue are increasingly recognized to play an important role in the regulation of glucose metabolism. However, much less is known about their effect on lipid metabolism. The fasting-induced adipose factor (FIAF/ angiopoietin-like protein 4/peroxisome proliferator-activated receptor γ angiopoietin-related protein) was previously identified as a target of hypolipidemic fibrate drugs and insulin-sensitizing thiazolidinediones. Using transgenic mice that mildly overexpress FIAF in peripheral tissues we show that FIAF is an extremely powerful regulator of lipid metabolism and adiposity. FIAF overexpression caused a 50% reduction in adipose tissue weight, partly by stimulating fatty acid oxidation and uncoupling in fat. In addition, FIAF overexpression increased plasma levels of triglycerides, free fatty acids, glycerol, total cholesterol, and high density lipoprotein (HDL)-cholesterol. Functional tests indicated that FIAF overexpression severely impaired plasma triglyceride clearance but had no effect on very low density lipoprotein production. The effects of FIAF overexpression were amplified by a high fat diet, resulting in markedly elevated plasma and liver triglycerides, plasma free fatty acids, and plasma glycerol levels, and impaired glucose tolerance in FIAF transgenic mice fed a high fat diet. Remarkably, in mice the full-length form of FIAF was physically associated with HDL, whereas truncated FIAF was associated with low density lipoprotein. In human both full-length and truncated FIAF were associated with HDL. The composite data suggest that via physical association with plasma lipoproteins, FIAF acts as a powerful signal from fat and other tissues to prevent fat storage and stimulate fat mobilization. Our data indicate that disturbances in FIAF signaling might be involved in dyslipidemia.

Obesity and associated diabetes mellitus type 2 have become major health concerns throughout the world. These ailments are intimately linked as excess weight greatly increases the likelihood of developing diabetes. Obesity also increases the risk for other clinical abnormalities such as hypertension and dyslipidemia. Although the positive associa-

tion between obesity and several co-morbidities has been well established at the epidemiological level, the mechanisms behind these associations are much less clear. Much attention has been given to the role of plasma free fatty acids (FFAs),³ which are elevated in the obese and able to disrupt cellular metabolism in several organs. However, it also has become evident that a variety of hormonal factors produced by adipose tissue can greatly affect organ functioning, especially at the metabolic and immunological levels (1). Indeed, these so-called adipocytokines or adipokines are now known to affect diverse biological processes, ranging from energy intake, insulin sensitivity, and hepatic glucose production, to reproductive and immunological function (2–5). Whereas the effects of adipocytokines on glucose homeostasis are becoming increasingly transparent, much less is known about how they regulate plasma lipid metabolism.

A relatively poorly characterized adipocytokine that may be involved in regulation of plasma lipid metabolism is the fasting-induced adipose factor (FIAF), also known as PPAR γ angiopoietin-related protein, angiopoietin-like protein 4, or hepatic fibrinogen/angiopoietin-related protein (6, 8–10). FIAF is a glycoprotein of ~50 kDa that belongs to the family of fibrinogen/angiopoietin-like proteins. In mice FIAF is most highly expressed in white and brown adipose tissue, and to a much lesser extent in other tissues such as heart, skeletal muscle, and liver (6, 8).

The most compelling data to date link FIAF with regulation of lipid metabolism. FIAF was first identified as a target gene of the nuclear receptors PPAR α and PPAR γ , which govern lipid metabolism in liver and white adipose tissue, respectively (6, 8). Subsequent studies employing adenoviral-mediated overexpression of FIAF or injection of recombinant FIAF have shown that FIAF potently elevates plasma triglyceride (TG) levels (10–12). This is possibly achieved by inhibition of lipoprotein lipase, the enzyme that is rate-limiting for plasma TG hydrolysis (10, 11, 13). According to a recent report, FIAF may also potently lower plasma glucose levels. In wild-type C57/B6 as well as in *db/db* mice, adenoviral-mediated overexpression of FIAF was found to improve hyperglycemia, hyperinsulinemia, and glucose tolerance (12).

In addition to lipid metabolism, FIAF has also been associated with angiogenesis. Expression of FIAF is up-regulated during hypoxia in both endothelial cells and cardiomyocytes, which probably occurs via the hypoxia-inducible factor 1 α (HIF-1 α) (14, 15). Furthermore, FIAF is

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³ The abbreviations used are: FFA, free fatty acid; FIAF, fasting-induced adipose factor; PPAR, peroxisome proliferator-activated receptor; TG, triglyceride; FIAF-Tg, FIAF transgenic; VLDL, very low density lipoprotein; HDL, high density lipoprotein; FPLC, fast protein liquid chromatography; LPL, lipoprotein lipase; WAT, white adipose tissue; BAT, brown adipose tissue; HSL, hormone-sensitive lipase; Q-PCR, quantitative real time PCR; HFD, high fat diet; LFD, low fat diet; ASP, acylation-stimulating protein.

expressed in certain tumors, especially in the hypoxic area surrounding necrotic cells, and has been suggested as a marker for conventional renal cell carcinoma. In the chicken chorioallantoic membrane assay, FIAF is able to induce a strong pro-angiogenic response (15). In contrast, others have ascribed a potent anti-angiogenic function to FIAF (16). Thus, the role of FIAF in angiogenesis remains ambiguous.

To determine the physiological role of FIAF, we studied the effect of FIAF overexpression in a transgenic mouse model. Our data indicate that FIAF, which is physically associated with plasma lipoproteins, is an important determinant of plasma TG concentration and clearance at physiological levels of expression. In addition, it stimulates adipose tissue lipolysis. In contrast to a recent study (12), in our model FIAF overexpression was associated with deterioration of glucose tolerance. Finally, we observed that the effects of FIAF overexpression were clearly amplified by feeding a high fat diet.

EXPERIMENTAL PROCEDURES

Generation of FIAF Transgenic (FIAF-Tg) Mice—The complete murine FIAF gene was amplified by PCR (High fidelity *Taq* polymerase, Roche Applied Science) from genomic DNA of mouse ES cells (Sv129) using primers CGGGCTCCAGATCTTCTTCTGCACCAG and GTCAGAGGCGGCATTGGACCCCTTGAAGTA and subcloned into the pGEM-Teasy vector (Promega, Leiden, the Netherlands). The proper sequence of the exons was verified by DNA sequencing. The mFIAF gene was subsequently cut out with *NotI* and placed behind the murine aP2 (adipocyte fatty acid-binding protein) promoter within pBS-SKII+ (kind gift of Dr. Bruce Spiegelman). The promoter plus the mFIAF gene were excised with *Clal* and *SacII* and gel purified before being injected into fertilized oocytes (strain FVB-Nlco, Eurogentec Transgenic Production Service, Seraing, Belgium).

Genotyping was performed on genomic DNA from mouse ears by Q-PCR (Sybr Green) using primers GCCCCATTGGTCACTCCTACAG and CGGCTCAGACTTAGACTTGCTC, which anneal within the aP2 promoter and the mFIAF gene, respectively, and control primers GCTGCTGGAGAATGAGTTGAATGC and CTCGGCTGAGTTTGAAGTTGAAGATG for the apoB gene.

Animal Experiments—All mice were on a pure-bred FVB-Nlco background. Male animals were kept in normal cages with food and water *ad libitum*. Mice in the fed state were sacrificed at the beginning of the light cycle. Mice in the fasted state were deprived of food for 6 h starting at the beginning of the light cycle. At the time of sacrifice animals were between 2 and 4 months of age. For the diet intervention, 2-month-old male mice were fed with a low or high fat diet for 10 weeks. The respective diets provided either 10 or 45% energy percent in the form of lard fat (D12450B or D12451, Research Diets, New Brunswick, NJ). Blood was collected via orbital puncture into EDTA tubes. Tissues were excised, weighed, and immediately frozen in liquid nitrogen.

Intragastric Lipid Loading Test—Mice fasted for 16 h were administered 350 μ l of olive oil (Bertolli, Extra Virgin) by intragastric gavage. Blood was collected by tail bleeding every 2 h for plasma TG measurement.

VLDL Production Test—Mice fasted for 16 h were injected via the tail vein with 500 mg/kg bodyweight Triton WR1339 (Tyloxapol) under general anesthesia. Blood was collected by tail bleeding at several time points during 2.5 h for plasma TG measurement.

Intraperitoneal Glucose Tolerance Test—After a 5-h fast mice were injected intraperitoneally with glucose (2 g/kg bodyweight on chow, 1 g/kg bodyweight on low fat/high fat diet). Blood was collected by tail bleeding after 0, 20, 40, 60, 90, and 150 min, and glucose was measured

using Accucheck compact (Roche Diagnostics, Almere, the Netherlands).

Insulin Tolerance Test—After a 5-h fast mice were injected intraperitoneally with insulin (0.75 unit/kg bodyweight). Blood was collected by tail bleeding after 0, 20, 40, and 60 min, and glucose was measured using Accucheck compact.

Plasma Metabolites—Plasma was obtained from blood by centrifugation for 10 min at $10,000 \times g$. The plasma glucose concentration was determined using a kit from Elitech (Sopachem, Wageningen, the Netherlands). Plasma and tissue triglycerides, plasma glycerol, and plasma total and HDL-cholesterol concentration were determined using kits from Instruchemie (Delfzijl, the Netherlands). Plasma free fatty acids were determined using a kit from WAKO Chemicals (Sopachem, Wageningen, the Netherlands).

Lipoprotein Profiling—Lipoproteins were separated using fast protein liquid chromatography (FPLC). 0.2 ml of pooled mouse plasma or human plasma was injected onto a Superose 6B 10/30 column (Amersham Biosciences) and eluted at a constant flow of 0.5 ml/min with PBS (pH 7.4). The effluent was collected in 0.5-ml fractions, and triglyceride and cholesterol levels were determined.

Plasma LPL and Hepatic Lipase Level Assay—Plasma lipoprotein lipase (LPL) and hepatic lipase levels were determined in post-heparin plasma as described before (17).

Q-PCR—Total RNA was extracted from tissues with TRIzol reagent (Invitrogen). 1 μ g of total RNA was reverse-transcribed with iScript (Bio-Rad). cDNA was PCR-amplified with Platinum *Taq* DNA polymerase (Invitrogen) on a Bio-Rad iCycler apparatus. Primers were designed to generate a PCR amplification product of 100–150 bp. Specificity of the amplification was verified by melt curve analysis and evaluation of efficiency of PCR amplification. Sequences of primers used are available on request.

Immunoblot—Immunoblotting on FPLC fractions, plasma, and tissues was carried out as described previously (6, 7). FIAF antibodies were directed against peptide epitopes within the N-terminal region of the mFIAF and hFIAF proteins.

Microarray—RNA was prepared from adipose tissue of 10 wild-type and 10 FIAF-Tg mice using TRIzol and subsequently pooled per group. Pooled RNA was further purified using Qiagen RNeasy columns and the quality verified by laboratory on a chip analysis (Bioanalyzer 2100, Agilent). 1 μ g of RNA was used for one cycle cRNA synthesis (Affymetrix, Santa Clara, CA). Hybridization, washing, and scanning of Affymetrix GeneChip mouse genome 430 2.0 arrays were carried out according to standard Affymetrix protocols. Fluorometric data were processed by Affymetrix GeneChip Operating software, and the gene chips were globally scaled to all the probe sets with an identical target intensity value. Further analysis was performed by Data Mining Tool (Affymetrix).

Ethical Considerations—The animal experiments were approved by the animal experimentation committee of Wageningen University. All human experiments were approved by the medical ethics committee of Wageningen University.

RESULTS

FIAF-Tg Mice Have Reduced Fat Mass—To investigate the role of FIAF in mammalian metabolism we generated transgenic mice that express mouse FIAF under the influence of the aP2 promoter (Fig. 1A), aiming at adipose tissue-specific overexpression (18). Two independent transgenic lines were obtained. Results presented here are from one transgenic line that showed up-regulation of mFIAF mRNA to levels similar to those achieved after fasting. FIAF mRNA was modestly up-regulated in white and brown adipose tissue and, unexpectedly, in skel-

FIGURE 1. FIAF is overexpressed in WAT of FIAF-Tg mice. *A*, schematic representation of the construct used for generating the FIAF-Tg mice. *B*, FIAF mRNA expression in WAT of fed wild-type and FIAF-Tg mice as determined by RNase protection assay. *C*, FIAF mRNA expression in WAT, BAT, liver, gastrocnemius, and heart of fed wild-type and FIAF-Tg mice as determined by Q-PCR ($n = 9$). Errors bars reflect \pm S.E. Differences between wild-type and FIAF-Tg mice were evaluated by Student's *t* test. **, $p < 0.01$; ***, $p < 0.001$. Protein expression of truncated FIAF in white adipose tissue (*D*) and blood plasma (*E*) of fed wild-type and FIAF-Tg mice. Equal amount of total protein was loaded per lane.

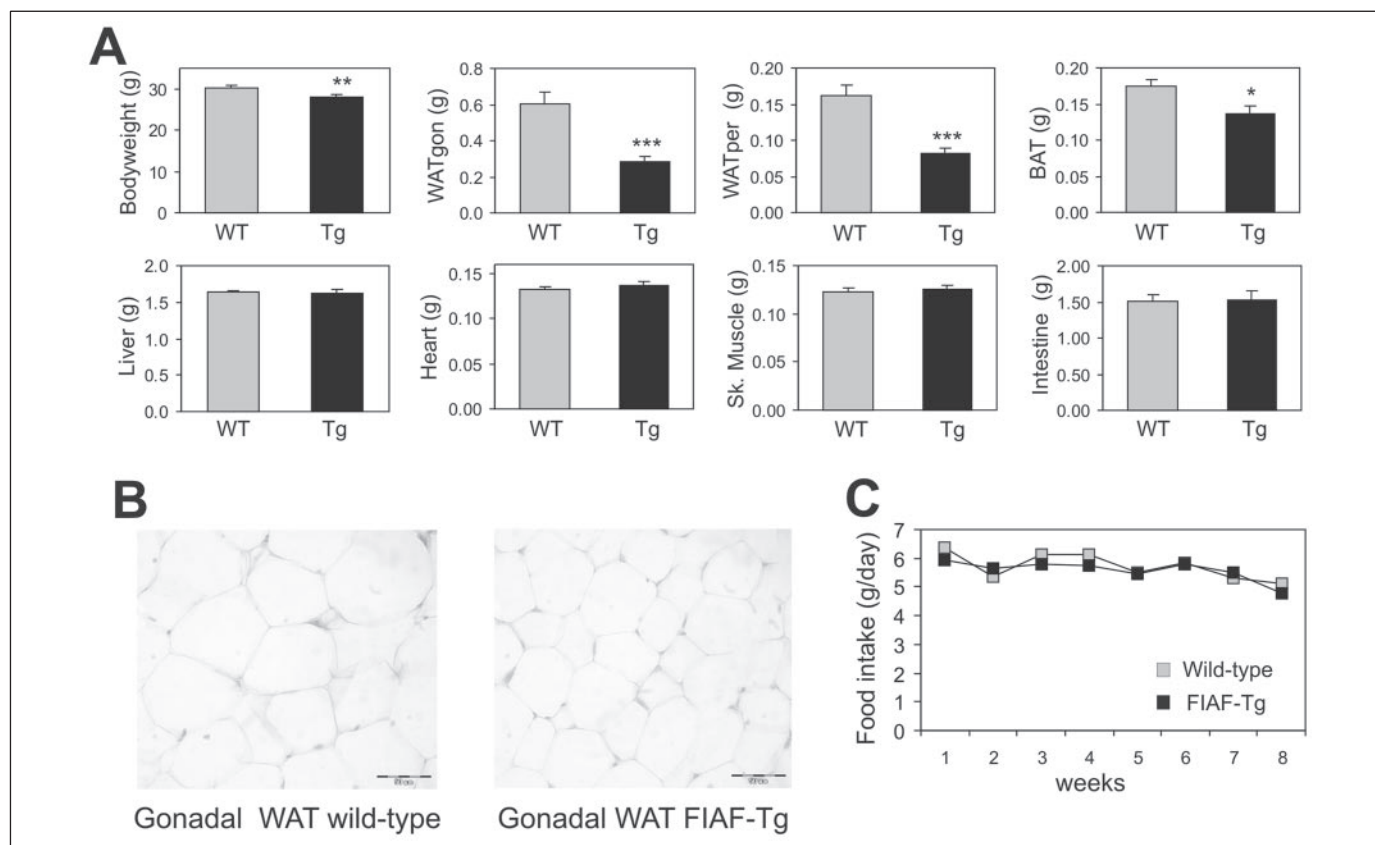
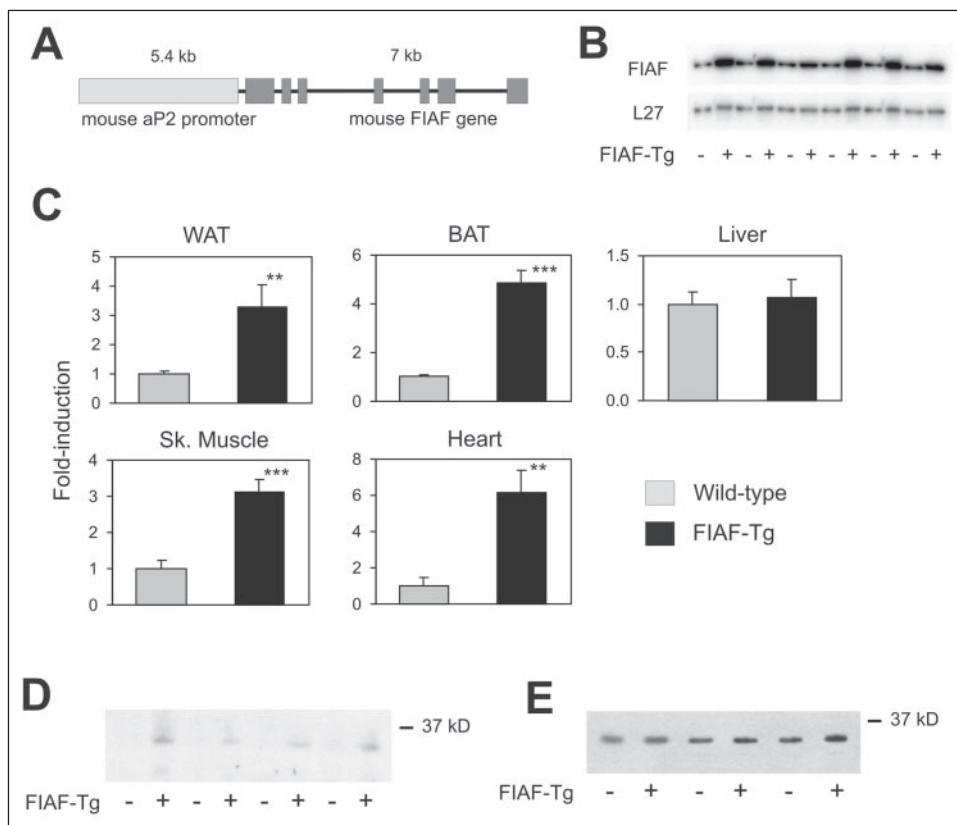


FIGURE 2. FIAF-Tg mice have markedly reduced fat mass. *A*, bodyweight, gonadal fat (WATgon), perirenal fat (WATper), brown adipose tissue (BAT), liver, heart, gastrocnemius, and small intestine weights of wild-type mice and FIAF-Tg mice ($n = 9$, matched according to litter). Error bars reflect \pm S.E. Differences between wild-type and FIAF-Tg mice were evaluated by Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$. *B*, eosin and hematoxylin staining of WAT of a representative wild-type and FIAF-Tg mouse. Bars indicate 50 μ m. *C*, daily food intake of wild-type mice (gray squares, $n = 9$) and FIAF-Tg mice (black squares, $n = 10$) as assessed over a period of 8 weeks.

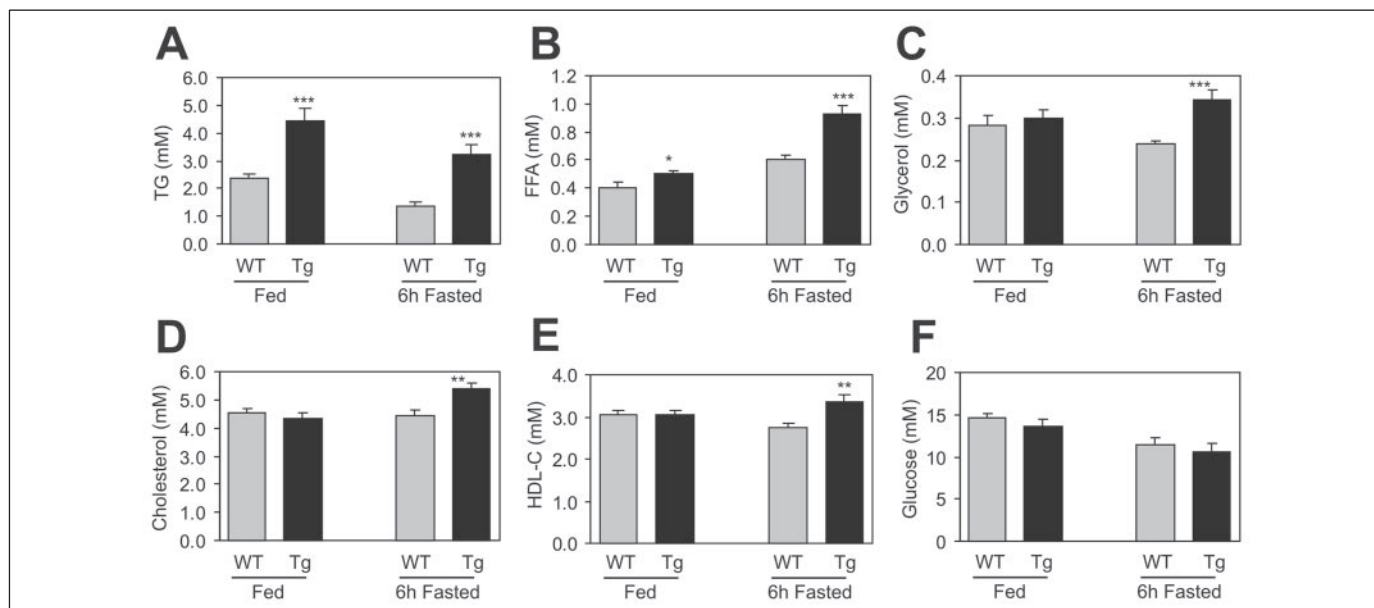


FIGURE 3. Fasting plasma triglycerides, free fatty acids, glycerol, HDL-cholesterol, and total cholesterol concentration are elevated in FIAF-Tg mice. Plasma TG (A), FFAs (B), glycerol (C), total cholesterol (D), HDL-cholesterol (E), and glucose (F), were determined in EDTA plasma of fed ($n = 9$) and 6-h-fasted ($n = 10$) wild-type mice (gray bars) and FIAF-Tg mice (black bars). Error bars reflect \pm S.E. Differences between wild-type and FIAF-Tg mice were evaluated by Student's *t* test. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

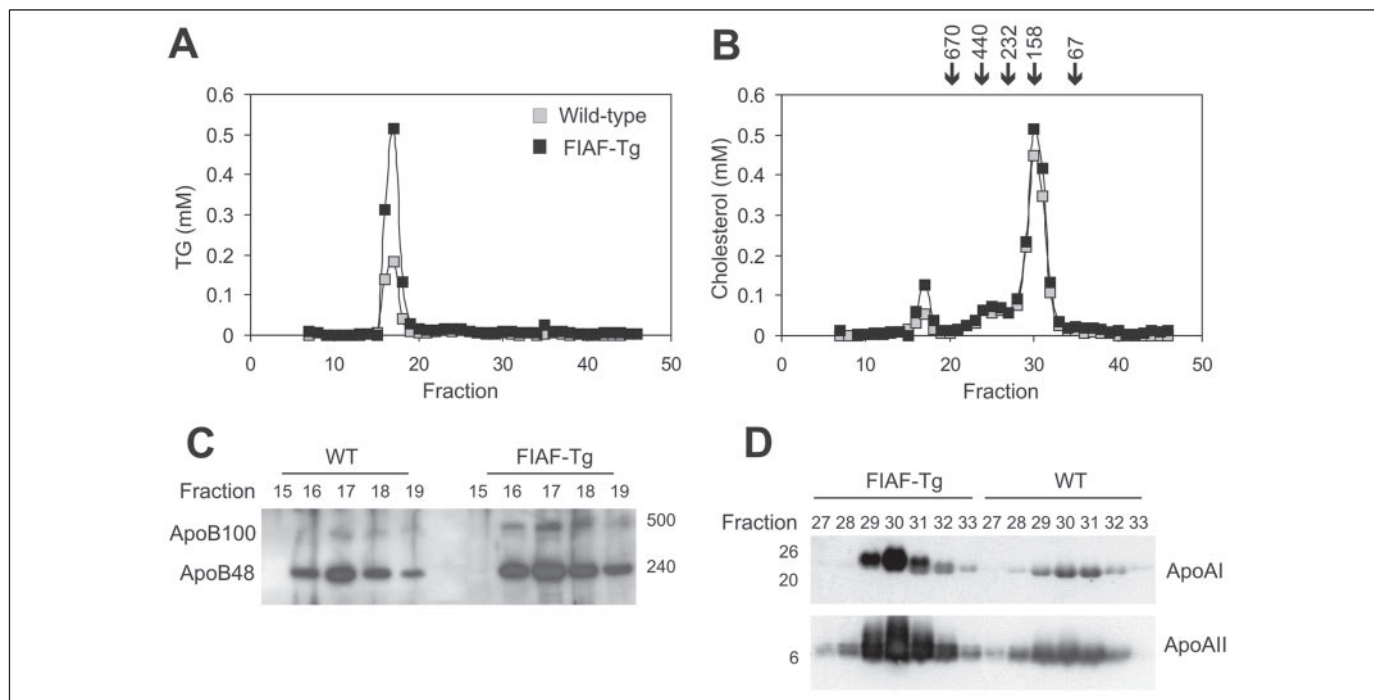


FIGURE 4. Fasting plasma VLDL and HDL concentrations are elevated in FIAF-Tg mice. Pooled plasma of 10 wild-type mice (gray squares) and 10 FIAF-Tg mice (black squares) fasted for 6 h was used for lipoprotein profiling by FPLC. Fractions were assayed for triglycerides (A), and cholesterol (B). Elution of protein markers is indicated. C, immunoblot of plasma lipoprotein FPLC fractions representing VLDL using antibody against apoB. D, immunoblot of plasma lipoprotein FPLC fractions representing HDL using antibody against apoA1 and apoAII.

etal muscle and heart, yet not in liver (Fig. 1, B and C). Activity of the transgene in skeletal muscle and heart is probably due to the design of the targeting vector, which contains the intact mouse FIAF gene. Previously, we showed that mFIAF gene expression is responsive to PPARs via a PPAR-responsive element within intron 3 (7). For reasons that are not clear this did not cause FIAF overexpression in liver. The difference in FIAF mRNA between wild-type and FIAF-Tg mice was translated at the protein level, as observed by immunoblot on adipose tissue using purified anti-mFIAF antibody (Fig. 1D). A modest increase in FIAF-S2

and FIAF-S1 proteins, which are the predominant and most easily detectable (truncated) forms of mFIAF in blood plasma (7), was observed in plasma of FIAF-Tg mice (Fig. 1E and data not shown).

Strikingly, FIAF-Tg mice weighed significantly less than their wild-type littermates (Fig. 2A). This was mostly due to a decrease in white adipose tissue (WAT) weight, which was $\sim 50\%$ lower in FIAF-Tg mice (Fig. 2A). In contrast, liver weight as well as the weight of numerous other organs was unaltered in the FIAF-Tg mice, whereas weight of brown adipose tissue (BAT) was modestly decreased. Reduced WAT

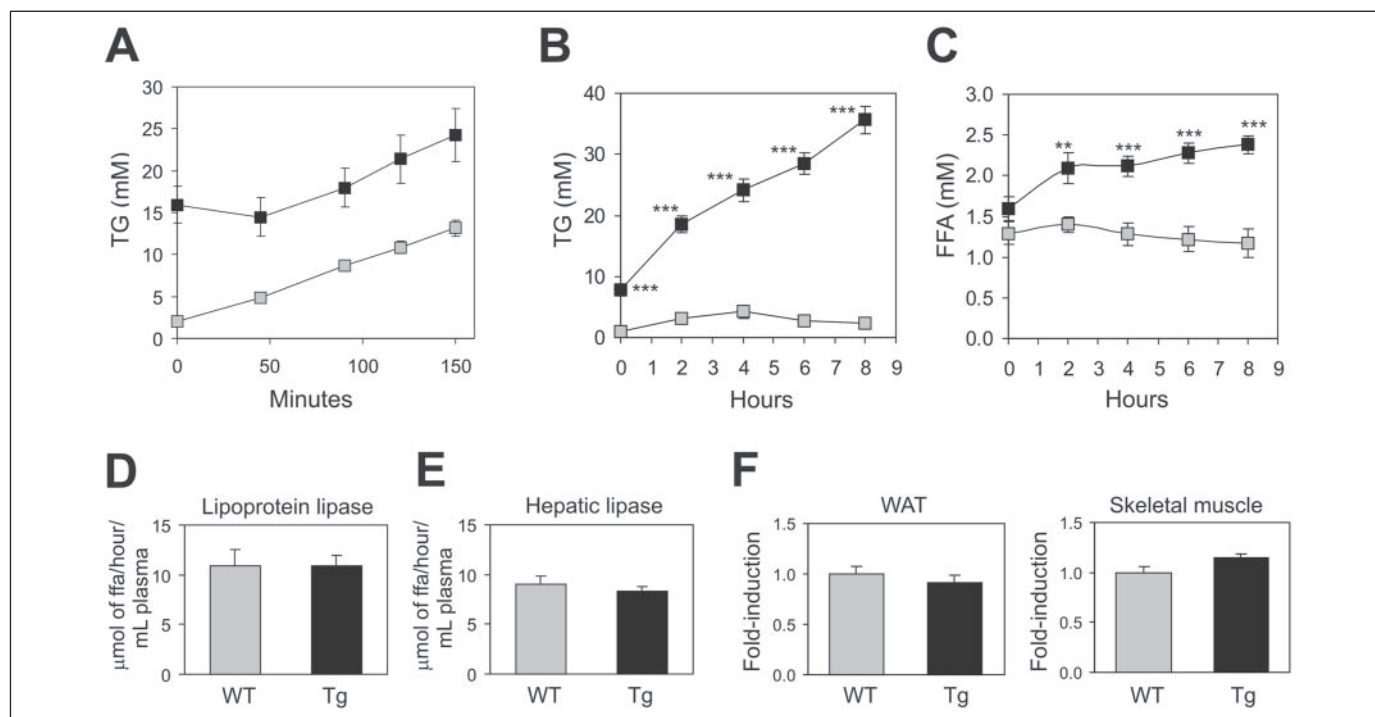


FIGURE 5. **Plasma TG clearance is severely impaired in FIAF-Tg mice.** A, VLDL production test in 16-h-fasted wild-type (gray squares, $n = 7$) and FIAF-Tg mice (black squares, $n = 8$). Plasma TG (B) or FFAs (C) during oral lipid loading test in 16-h-fasted wild-type (gray squares, $n = 7$) and FIAF-Tg mice (black squares, $n = 9$). In vitro lipoprotein lipase (D) and hepatic lipase (E) activity was measured in pre- and post-heparin plasma of 5-h-fasted wild-type ($n = 5$) and FIAF-Tg mice ($n = 8$). F, mRNA expression of lipoprotein lipase in WAT or gastrocnemius of wild-type and FIAF-Tg mice ($n = 9$), as determined by Q-PCR. Differences between wild-type and FIAF-Tg mice were evaluated by Student's *t* test. **, $p < 0.01$; ***, $p < 0.001$.

weight was due to a decrease in adipocyte size (Fig. 2B). Reduced adiposity was not the result of diminished food intake, which was identical between the two sets of mice (Fig. 2C). These data indicate that FIAF overexpression results in loss of body fat.

FIAF-Tg Mice Display Hypertriglyceridemia and Hypercholesterolemia—To get a better understanding of the potential cause of the reduced adiposity, plasma lipid parameters were assessed in both fed and 6-h-fasted mice. A marked increase in plasma TG levels was observed in the FIAF-Tg mice, which was independent of their feeding status (Fig. 3A). In addition, plasma FFAs, glycerol, total cholesterol, and HDL-cholesterol concentrations were significantly elevated in the FIAF-Tg mice in the fasted state, whereas glucose was unchanged (Fig. 3, B–F). Thus, FIAF overexpression has profound effects on plasma lipid concentrations. Profiling of lipoproteins using FPLC analysis revealed that the increase in plasma TG in the 6-h-fasted animals was attributable to the VLDL fractions (Fig. 4A), whereas the increase in cholesterol was found in both VLDL and HDL fractions (Fig. 4B). Immunoblotting demonstrated a marked increase in apoB48 and apoB100 protein content in the VLDL fractions in FIAF-Tg mice, indicating that the number of VLDL particles in plasma is increased in these mice (Fig. 4C). It should be emphasized that in mice apoB48 accounts for approximately two-thirds of total apoB production in liver. Similarly, we found markedly increased apoAI and apoAII protein content in the HDL fractions in FIAF-Tg mice, pointing toward a pronounced increase in the number of HDL particles (Fig. 4D).

Elevated plasma TG levels in 6-h-fasted mice can either be due to increased VLDL production or impaired clearance. To discriminate between these two possibilities, a VLDL production test and an oral lipid-loading test were performed. Despite greatly elevated 16-h fasting plasma TG levels in FIAF-Tg mice, no difference in VLDL production, determined by the slope of increase of plasma TG after injection with Triton WR1339, was observed between wild-type and FIAF-Tg mice (Fig. 5A). In contrast, an oral lipid loading test revealed dramatic differ-

ences between the two sets of mice. Whereas in wild-type mice intra-gastric loading of olive oil caused a moderate and transient increase in plasma TG, in FIAF-Tg mice plasma TG went up dramatically, reaching levels of almost 35 mM after 8 h (Fig. 5B). Post-prandial plasma FFAs were also significantly increased in FIAF-Tg mice (Fig. 5C). These data show unambiguously that clearance of TG-rich apoB-containing lipoproteins is severely inhibited by FIAF overexpression. Impaired clearance of plasma TG can be caused by decreased presence of lipoprotein lipase (LPL) and/or inhibition of LPL activity. To investigate whether total LPL content was altered, total hepatic lipase and LPL levels were determined in post-heparin plasma. Plasma levels of these lipases were very similar between wild-type and FIAF-Tg mice (Fig. 5, D and E). In addition, mRNA expression and tissue protein level of LPL in adipose tissue or skeletal muscle were not affected by FIAF overexpression (Fig. 5F and data not shown). This suggests that FIAF may block *in vivo* LPL activity, rather than reduce total LPL level. This notion is backed up by evidence showing inhibition of LPL activity by recombinant FIAF (10). Together, these data indicate that FIAF overexpression markedly impairs plasma TG clearance and accordingly raises plasma TG levels, most likely via inhibition of LPL activity.

FIAF Is Physically Associated with HDL—Several proteins that are physically associated with plasma lipoproteins, including apoCs and apoAV, are known to influence LPL activity. Because FIAF overexpression increases fasting plasma VLDL and HDL levels, we hypothesized that FIAF may be physically associated with VLDL or HDL. To determine whether this is the case, immunoblotting was performed on the corresponding mouse plasma FPLC fractions using a well characterized anti-mFIAF antibody (6). Remarkably, it was observed that full-length FIAF was present in the HDL fractions, but not in the VLDL fractions (Fig. 6A). A small band, corresponding to truncated FIAF-S2 (7), was visible in the first HDL fraction that overlaps with LDL. Further analysis

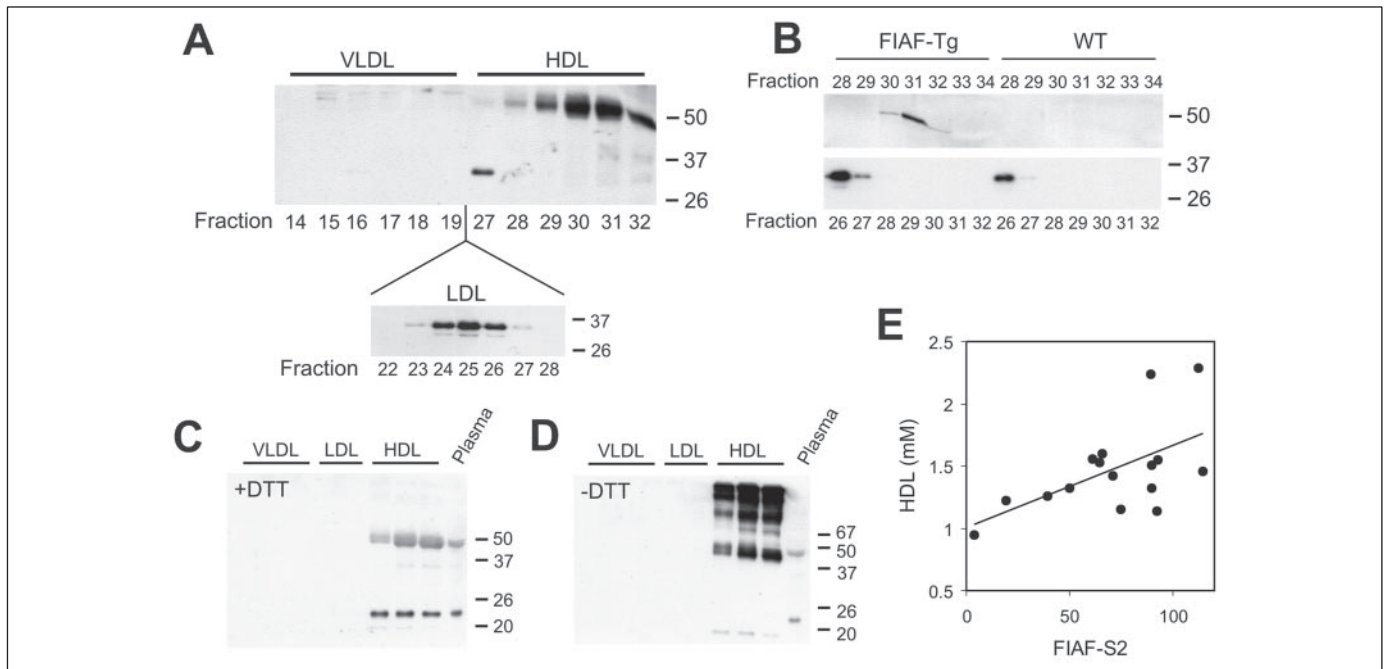


FIGURE 6. FIAF is physically associated with plasma lipoproteins. *A*, immunoblot of mouse plasma lipoprotein FPLC fractions using antibody against mFIAF (6). *B*, immunoblot of plasma lipoprotein FPLC fractions representing HDL of wild-type and FIAF-Tg mice using antibody against mFIAF. *C* and *D*, immunoblot of human plasma lipoprotein FPLC fractions using antibody against hFIAF (7). The loading buffer was with (*C*) or without (*D*) dithiothreitol (DTT). Human plasma was used to mark full-length and truncated FIAF (loading buffer used was with dithiothreitol). *E*, correlation between relative plasma levels of truncated FIAF (FIAF-S2), as determined by immunoblot, and HDL-cholesterol concentration in 16 healthy young adults ($r = 0.57$, $p = 0.01$).

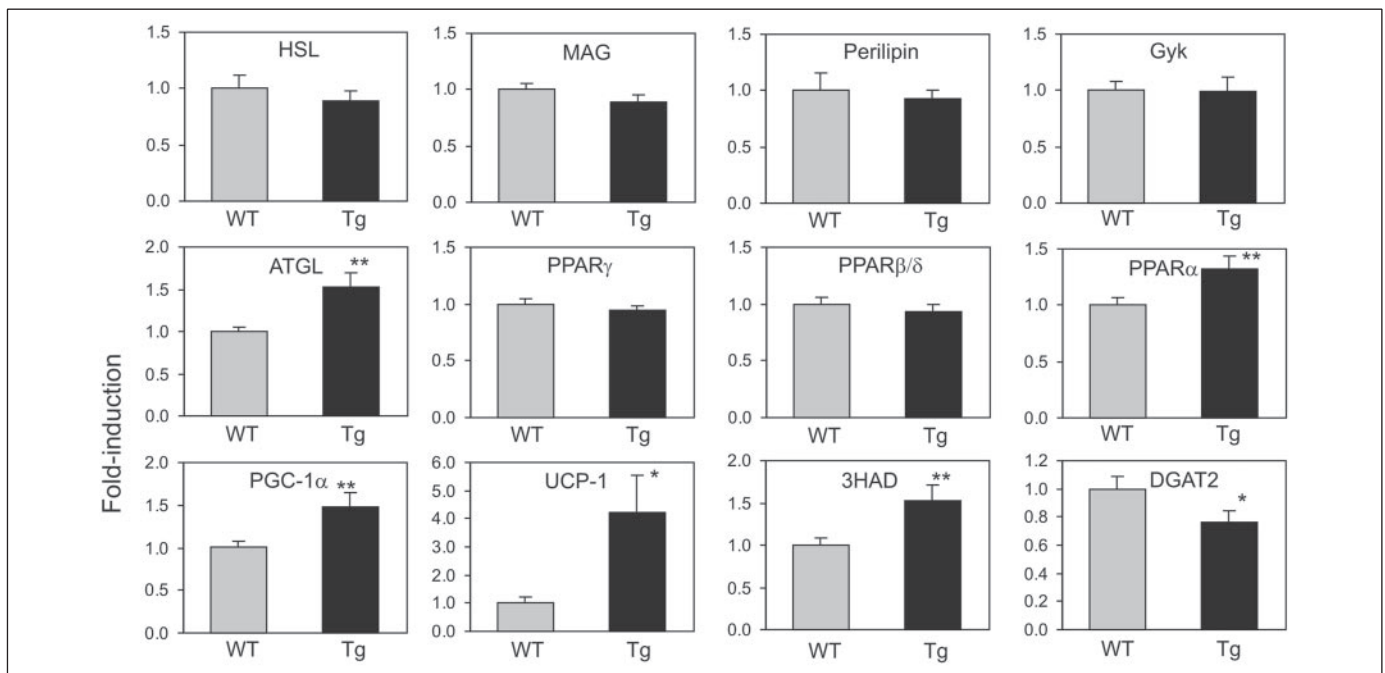


FIGURE 7. mRNA expression of selected genes in WAT of wild-type and FIAF-Tg mice. Gene expression was determined by Q-PCR. Errors bars reflect S.E. ($n = 9$). Differences between wild-type and FIAF-Tg mice were evaluated by Student's *t* test. *, $p < 0.05$; **, $p < 0.01$.

clearly demonstrated that truncated FIAF is present in the LDL fractions. Both full-length and truncated FIAF were more abundant in the HDL and LDL fractions, respectively, of FIAF-Tg mice (Fig. 6*B*). Remarkably, in human plasma full-length FIAF, truncated FIAF and FIAF multimers, the latter visualized by omitting dithiothreitol from the loading buffer, were associated exclusively with HDL (Fig. 6, *C* and *D*). Together, the data indicate that in mice the full-length form of FIAF is

physically associated with HDL, whereas truncated FIAF is associated with LDL. In human both full-length and truncated FIAF are associated with HDL. The latter observation suggests that plasma FIAF and HDL levels may be correlated. Indeed, in a group of 16 healthy young subjects we found a significant positive correlation ($r = 0.57$, $p = 0.01$) between plasma levels of FIAF-S2 and HDL (Fig. 6*E*). In contrast, no significant correlation was found for plasma TG or LDL (data not shown).

FIAF Governs Plasma Lipid Levels and Adiposity

Gene Expression Changes in WAT of FIAF-Tg Mice Indicate Enhanced Lipolysis and Oxidative Metabolism—The parallel increase in plasma FFAs and glycerol in FIAF-Tg mice cannot be explained by inhibition of plasma TG catabolism but rather is indicative of enhanced adipose tissue lipolysis. Lipolysis is catalyzed by hormone-sensitive lipase (HSL), which is tightly regulated by numerous external stimuli, including insulin and β -adrenergic activity. mRNA expression of HSL was not altered in the FIAF-Tg mice, nor that of other genes that could affect plasma FFA and glycerol concentrations, including monoglyceride lipase, perilipin, PPAR γ , and glycerol kinase (Fig. 7). Interestingly, expression of adipose triglyceride lipase (ATGL)/desnutrin, a recently discovered adipose tissue lipase that works in conjunction with HSL (19, 20), was 50% increased in the FIAF-Tg mice. Accordingly, the elevated plasma FFA and glycerol levels are likely caused by enhanced lipolysis possibly via up-regulation of ATGL/desnutrin.

After hydrolysis of plasma TG by LPL, the fatty acids that enter the adipocyte can either be reconverted into triglycerides and stored as such, or can be oxidized for energy. Interestingly, expression of the co-activator PPAR γ co-activator 1 α (PGC-1 α) and the nuclear receptor PPAR α , both of which are involved in oxidative metabolism, were significantly up-regulated in WAT of FIAF-Tg mice (Fig. 7). Ectopic expression of PGC-1 α in WAT has been shown to stimulate oxidative metabolism and expression of the uncoupling protein 1, whereas PPAR α is known to up-regulate a whole spectrum of genes involved in the fatty acid oxidative pathway (21, 22). Accordingly, we observed that expression of uncoupling protein 1 was almost 5-fold elevated in WAT of FIAF-Tg mice (Fig. 7). Furthermore, Affymetrix microarray analysis, performed in single replicates and confirmed by Q-PCR, indicated that the expression of numerous PPAR α target genes involved in fatty acid oxidation were modestly up-regulated in WAT of FIAF-Tg mice (Table 1). Expression of PPAR β/δ was unchanged, whereas diacylglycerol acyltransferase 2, which is involved in TG synthesis and the most highly expressed diacylglycerol acyltransferase in adipose tissue, was significantly down-regulated in FIAF-Tg mice. Thus, FIAF overexpression causes changes in gene expression in WAT consistent with preferential oxidation of fatty acids at the expense of storage.

FIAF-Tg Mice Display Impaired Glucose Tolerance—Chronic elevation of plasma FFAs has been associated with impaired control of plasma glucose. Indeed, we found that glucose tolerance was mildly but significantly deteriorated in FIAF-Tg mice (Fig. 8A), whereas the sensitivity to exogenous insulin was not different (Fig. 8B). It should be noted that in FVB mice, which is the background strain for the FIAF-Tg mice, glucose tolerance is already poor under normal conditions. Impaired glucose tolerance was not due to accumulation of TG in liver and skeletal muscle, which were unaltered in FIAF-Tg mice (Fig. 8, C and D).

High Fat Feeding-induced Fatty Liver, Hypertriglyceridemia, and Glucose Intolerance Are More Pronounced in FIAF-Tg Mice—Taking into account the inhibitory effect of FIAF on plasma TG catabolism, it can be expected that the effects of FIAF overexpression become much more severe under conditions of fat overload such as high fat feeding. To determine whether this is the case, wild-type and FIAF-Tg mice were fed a high fat diet (HFD) or low fat diet (LFD) for 10 weeks. Whereas HFD had little effect on plasma TG in wild-type mice, it markedly elevated plasma TG in FIAF-Tg mice (Fig. 9A). Furthermore, whereas HFD had little effect on glucose tolerance in wild-type FVB mice, which are known to be resistant to diet-induced obesity/glucose intolerance (23, 24), it caused a significant deterioration of glucose tolerance in FIAF-Tg mice (Fig. 9B). A similar genotype-specific effect of HFD was observed for plasma FFA and glycerol levels, indicating elevated adipose tissue lipolysis (Fig. 9, C and D). Also, plasma HDL levels were significantly

TABLE 1

Gene expression profiling (Affymetrix) of WAT of wild-type and FIAF-Tg mice

Selected genes involved in energy metabolism are shown.

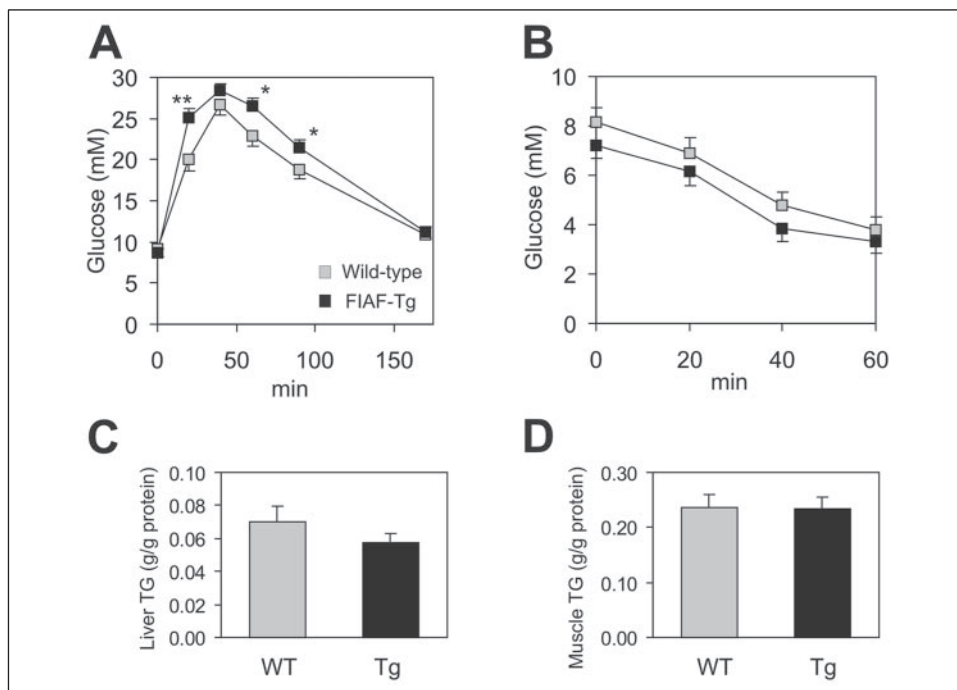
Probe set ID	Gene	Fold-increase	Regulated by PPAR α
1417130_s_at	<i>Angptl4</i>	1.62	x
Mitochondrial and peroxisomal fatty acid oxidation			
1448382_at	<i>Ehhadh</i>	1.55	x
1435630_s_at	<i>Acat2</i>	1.40	x
1425195_a_at	<i>Acat2/Acat3</i>	1.38	x
1423108_at	<i>Slc25a20</i>	1.36	x
1424184_at	<i>Acadvl</i>	1.35	x
1418911_s_at	<i>Acs14</i>	1.35	x
1448286_at	<i>Hadh2</i>	1.32	x
1452341_at	<i>Echs1</i>	1.28	x
1423797_at	<i>Aacs</i>	1.27	
1418321_at	<i>Dci</i>	1.25	x
Eicosanoid metabolism			
1417263_at	<i>Ptgs2</i>	3.20	
1417262_at	<i>Ptgs2</i>	2.68	
1419905_s_at	<i>Hpgd</i>	1.36	x
1417777_at	<i>Ltb4dh</i>	1.26	
Fatty acid synthesis			
1430307_a_at	<i>Mod1</i>	1.36	x
1428190_at	<i>Slc25a1</i>	1.34	
1425326_at	<i>Acly</i>	126	
Transporter proteins			
1444489_at	<i>Slc25a12</i>	2.20	
1422906_at	<i>Abcg2</i>	1.96	x
1422811_at	<i>Slc27a1</i>	1.74	x
1426340_at	<i>Slc1a3</i>	1.74	
1440379_at	<i>Slc1a5</i>	1.65	
1437052_s_at	<i>Slc2a3</i>	1.39	
Amino acid metabolism			
1417772_at	<i>Grhpr</i>	1.30	x
1418847_at	<i>Arg2</i>	1.54	x
Nuclear receptor co-activators/repressors			
1460336_at	<i>Ppargc1a</i>	1.84	
1423201_at	<i>Ncor1</i>	1.38	
Miscellaneous involved in metabolism			
1437864_at	<i>Adipor2</i>	1.82	
1433691_at	<i>Ppp1r3c</i>	1.72	
1417956_at	<i>Cidea</i>	1.71	
1438258_at	<i>Vldlr</i>	1.60	x
1425631_at	<i>Ppp1r3c</i>	1.54	
1448499_a_at	<i>Ephx2</i>	1.42	x
1427213_at	<i>Pfkfb1</i>	1.44	x
1456090_at	<i>Pdhx</i>	1.40	
1416371_at	<i>Apod</i>	1.36	
1448172_at	<i>Mdh1</i>	1.23	

more elevated by HFD in FIAF-Tg mice compared with wild-type mice (Fig. 9E). Impaired plasma TG catabolism coupled with elevated adipose lipolysis is expected to increase fat delivery to the liver. Indeed, the HFD-induced elevation of plasma lipid levels in FIAF-Tg mice was associated with TG accumulation in liver, as assessed by quantitative and histological assays (Fig. 9, F and G). Elevated liver TG was probably not due to increased endogenous synthesis, because the lipogenic genes stearoyl-CoA desaturase 1 and fatty acid synthase were similarly down-regulated by HFD in wild-type and FIAF-Tg mice (Fig. 9H). Taken together, these data indicate that HFD magnifies the effects of FIAF overexpression on plasma lipid metabolism. Under these conditions, by preventing plasma TG catabolism and stimulating adipose tissue lipolysis, FIAF increases the flux of lipid to the liver leading to steatosis.

DISCUSSION

In the past few years, it has become increasingly clear that adipocytokines play a very important role in linking excess adipose tissue to impairment of glucose homeostasis. Indeed, several adipocytokines have been shown to profoundly affect peripheral and hepatic insulin sensitivity. However, limited data exist on the effect of adipocytokines

FIGURE 8. Glucose tolerance is modestly impaired in FIAF-Tg mice. Intraperitoneal glucose tolerance test (A), and insulin sensitivity test (B) were performed in 5-h-fasted wild-type mice ($n = 7$, gray squares) and FIAF-Tg mice ($n = 10$, black squares). Triglyceride concentration in liver (C) and gastrocnemius muscle (D) of fed wild-type mice (gray bars, $n = 9$) and FIAF-Tg mice (black bars, $n = 9$). Errors bars reflect \pm S.E. Differences between wild-type and FIAF-Tg mice were evaluated by Student's t test. *, $p < 0.05$; **, $p < 0.01$.



on plasma lipid and lipoprotein metabolism. The exception is acylation-stimulating protein (ASP), which is produced by adipose tissue and influences plasma TG metabolism. Studies in mice lacking the complement component 3 gene *C3*, which is the precursor for ASP, as well as studies with mice injected with recombinant ASP have shown that ASP stimulates uptake and storage of plasma TG in fat cells (25, 26). As opposed to ASP, our and other data show that in mice FIAF is a powerful inhibitor of plasma TG clearance. In addition, in adipose tissue FIAF stimulates lipolysis, resulting in elevated plasma FFA and glycerol levels, and may promote fatty acid oxidative metabolism and uncoupling. One major consequence of this action is diminished fat stores.

As the rate-controlling step in plasma TG clearance is catalyzed by LPL, FIAF most likely increases plasma TG levels in mice by blocking LPL, which is supported by *in vitro* studies using purified LPL and FIAF (10). The inhibitory effect of FIAF on LPL is similar to that of apoCI and apoCIII, and opposes that of apoCII and apoAV, the latter of which, interestingly, is also physically associated with HDL. Recent data suggest that apoAV lowers plasma TG by directing TG-rich lipoproteins to proteoglycans-bound LPL (27). However, it is not clear whether the physical association of FIAF with HDL is required for its hypertriglyceridemic effect. It can be speculated that inhibition of LPL is actually mediated by truncated FIAF bound to LDL. A mechanism opposite to apoCII but having the same net effect can be envisioned, where FIAF, rather than dissociating from VLDL as it decreases in size, associates with IDL/LDL and inhibits LPL activity to prevent complete TG hydrolysis. If inhibition of LPL by FIAF and thus its hypertriglyceridemic effect is truly dependent on its binding to LDL, FIAF may function differently in human, as in human FIAF is associated exclusively with HDL. An additional possible explanation for the impaired LPL activity in FIAF-Tg mice may be elevated plasma FFAs, which have been shown to potently inhibit LPL activity via numerous mechanisms (28). In the FIAF-Tg mice, plasma FFAs were elevated in fed and fasted state, as well as during the lipid loading test. Taken together, the inhibitory effect of FIAF on plasma TG clearance is likely explained by inhibition of LPL, although the one or more molecular and structural mechanisms behind this inhibition remain somewhat ambiguous.

Inhibition of LPL cannot be the cause of the elevated HDL-cholesterol levels in FIAF-Tg mice. Because LPL is structurally highly similar to hepatic lipase and endothelial lipase, and because impaired activity of these lipases causes elevation of plasma HDL (29–32), it is conceivable that FIAF may target hepatic lipase and endothelial lipase as well. Additional research is necessary to better define the molecular mechanisms behind the modestly elevated plasma HDL-cholesterol and markedly elevated plasma apoAI and apoAII levels in FIAF-Tg mice.

The increased levels of plasma FFAs and glycerol in FIAF-Tg mice are in agreement with the acute elevation in plasma FFAs observed after FIAF injection (10), indicating that FIAF stimulates adipose tissue lipolysis. We did not find any effect of FIAF on HSL mRNA expression, although we cannot rule out activation of HSL activity via an alternative mechanism. In contrast, the mRNA level of the recently described adipose triglyceride lipase (ATGL) was elevated by 50% in FIAF-Tg mice (19, 20). Thus, FIAF may stimulate lipolysis via up-regulation of ATGL.

Previous studies have shown that adenoviral-mediated overexpression of FIAF or injection of recombinant FIAF potently elevates plasma triglyceride levels (10, 11). Although these studies established FIAF as a modulator of plasma TG levels in mice, they did not allow appraisal of the impact of physiological changes in FIAF expression. What is remarkable about the present study is that a modest increase in FIAF mRNA in peripheral tissues is sufficient to cause fasting plasma TG to go up to 2.5- to 8-fold, depending on the duration of fasting. Coupled with the severely impaired plasma TG clearance after lipid loading, this indicates that FIAF is an extremely powerful regulator of plasma TG levels in mice.

In the FIAF-Tg mice impaired plasma TG clearance can be expected to diminish adipose stores (33). However, the fate of the excess TG remains unclear, because they do not appear to be stored in non-adipose tissues, at least under conditions of low fat diet. Based on our gene expression data, which show increased expression of a variety of genes involved in fatty acid oxidative metabolism and uncoupling, including PGC-1 α and uncoupling protein 1, we speculate that these extra TG were actually oxidized. Detailed indirect calorimetric studies will be necessary to clarify this issue.

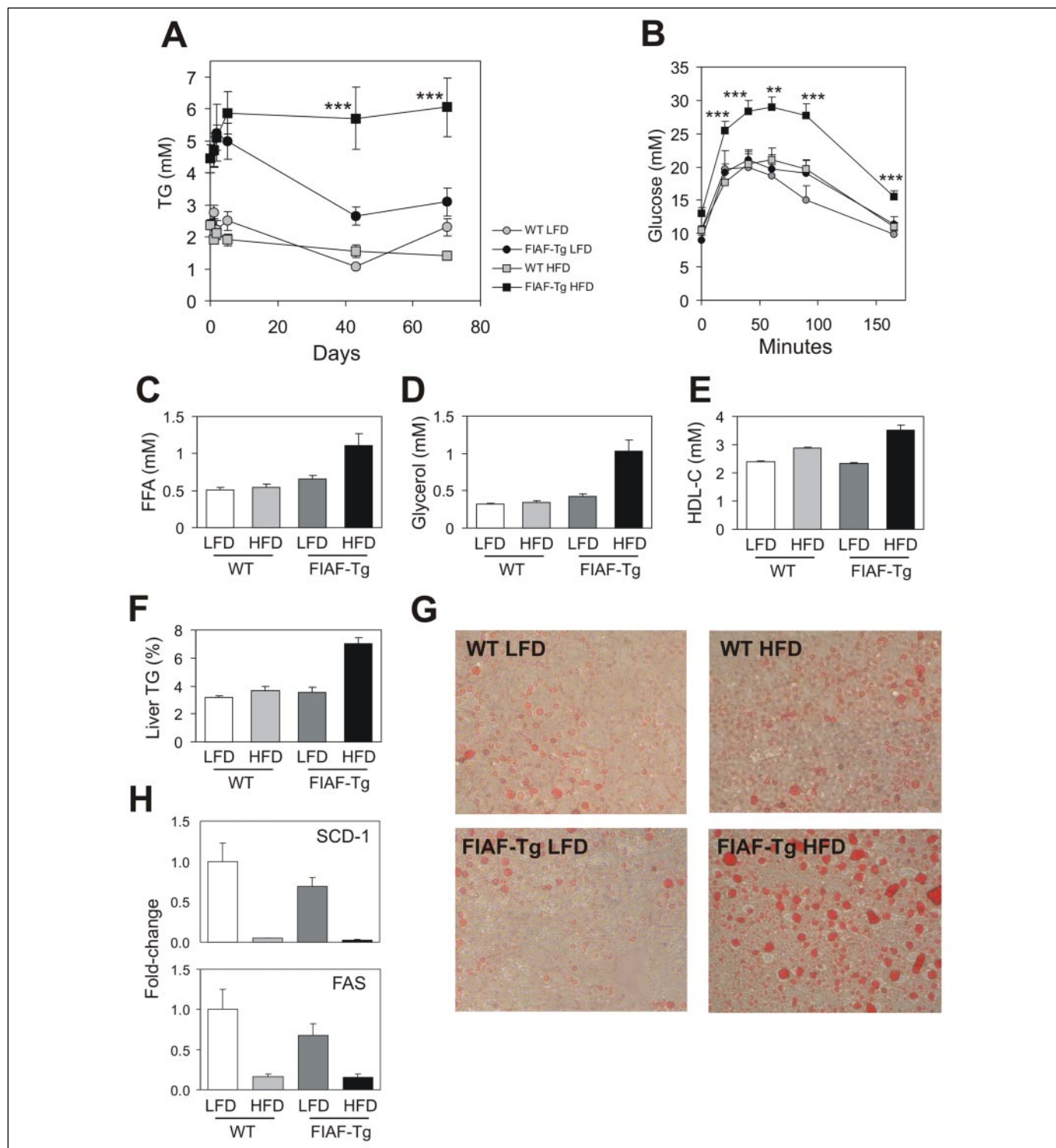


FIGURE 9. High fat diet magnifies the effect of FIAF-overexpression on lipid metabolism. A, time course of plasma TG in wild-type and FIAF-Tg mice during high fat (HFD) or low fat (LFD) feeding. B, intraperitoneal glucose tolerance test in wild-type and FIAF-Tg mice after 9 weeks of high fat or low fat feeding. Differences between wild-type and FIAF-Tg mice on HFD were evaluated by Student's *t* test. **, $p < 0.01$; ***, $p < 0.001$. Plasma FFA (C), glycerol (D), HDL-C (E), and liver TG (F) in wild-type and FIAF-Tg mice fed a LFD or HFD for 10 weeks. Significant effects were observed by two-way analysis of variance for diet (FFA, $p < 0.05$; glycerol, $p < 0.001$; HDL, $p < 0.0001$; liver TG, $p < 0.0001$), for genotype (FFA, $p < 0.0001$; glycerol, $p < 0.0001$; liver TG, $p < 0.0001$), and for the interaction between the two parameters (FFA, $p < 0.01$; glycerol, $p < 0.0001$; HDL, $p < 0.01$; liver TG, $p < 0.0001$). Error bars represent \pm S.E. G, histological sections of liver stained for lipids using Oil Red O. H, mRNA expression of lipogenic genes stearoyl-CoA desaturase (SCD-1) and fatty acid synthase (FAS) in liver, as determined by Q-PCR. The numbers of animals per group were: WT_LFD: 9; WT_HFD: 11; FIAF-Tg_LFD: 9; and FIAF-Tg_HFD: 7.

The present study clearly demonstrates that the effects of FIAF overexpression are much more pronounced in mice fed a high fat diet. In wild-type FVB mice, which are known to be resistant to high fat diet-induced obesity/glucose intolerance, high fat feeding had little effect on

plasma TG, FFAs, and glycerol concentration, as well as on glucose intolerance, but markedly augmented these parameters in mice overexpressing FIAF. Previously we showed that high fat feeding down-regulated plasma FIAF levels in mice (6), which in light of the present find-

ings may serve to facilitate the clearance of plasma TG into peripheral tissues, and thus minimize their hepatic and plasma accumulation. FIAF overexpression overruled the down-regulation, resulting in markedly elevated plasma TG levels and a fatty liver.

Recently, Xu *et al.* (12) reported that adenoviral-mediated overexpression of FIAF causes a pronounced decrease in plasma glucose level and improves glucose tolerance. In contrast, we show that FIAF overexpression has no effect on plasma glucose levels and impairs glucose tolerance, particularly after feeding a high fat diet. The reason for this discrepancy is not clear but may be related to the level of overexpression, which was modest in our experiments, as well as the site of overexpression (liver *versus* peripheral tissues). We previously demonstrated that human liver produces the truncated form of FIAF, whereas human adipose tissue only produces the full-length form (7). It can be hypothesized that these forms may have different activities toward glucose and lipid metabolism, similar to what has been demonstrated for globular and full-length adiponectin (34). It should be mentioned that, while our manuscript was in revision, Köster *et al.* (35) showed no effect of liver-specific hFIAF overexpression or general FIAF deletion on plasma glucose levels. In agreement with Xu *et al.* we find accumulation of hepatic triglycerides by FIAF overexpression, yet this is only observed after a high fat diet. The hepatic steatosis together with the markedly elevated plasma FFA levels may contribute to the impaired glucose tolerance in FIAF-Tg mice on a high fat diet.

An important question that is still not fully resolved is whether FIAF only acts locally in the tissue where it is produced, exerting an autocrine or paracrine action, or whether it also targets distant organs. Another key question is whether the effects of FIAF on lipid and glucose metabolism are exclusively mediated by inhibition of LPL or involve an additional mechanism. Blocking LPL is expected to result in a decreased plasma FFA level, rather than an increase as observed here and in another study (10), suggesting that FIAF acts in multiple ways. It can be hypothesized that in analogy to numerous other adipocytokines FIAF is able to bind a specific cell-surface receptor, although this remains to be demonstrated.

Although FIAF is most highly expressed in adipose tissue, it may exert important effects in other tissues as well. Recently, it was reported that down-regulation of FIAF expression in the intestine is essential for the increase in adipose mass induced by intestinal microbiota (36). The data suggested that FIAF is an important mediator of the physiological effects of the intestinal microbiota.

Despite bearing a name that belies any link with metabolism, in recent years it has become apparent that angiopoietin-like proteins are a group of proteins that play an important role in the regulation of lipid and glucose metabolism. Recent studies indicate that overexpression or disruption of Angptl3 and angiopoietin-related growth factor (Angptl6), which are mainly produced by the liver, result in major disturbances at the level of lipid metabolism and energy homeostasis (37–39). Accordingly, angiopoietin-like proteins have become very interesting targets for the pharmacological treatment of dyslipidemia and obesity.

In conclusion, FIAF represents a powerful signaling molecule from fat and other tissues that prevents plasma TG clearance and stimulates adipose TG mobilization. Further studies, including investigation of the connection between human plasma FIAF level and post-prandial TG response, are necessary to better define the role of FIAF in lipid metabolism in humans. Nevertheless, it can be speculated that FIAF may be involved in connecting adipose tissue to regulation of plasma lipid levels, and that changes in FIAF signaling may contribute to development of (diabetic) dyslipidemia.

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