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The role of mitochondrial glycerol-3-phosphate acyltransferase-1 in regulating lipid and glucose homeostasis in high-fat diet fed mice

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ABSTRACT

Glycerol-3-phosphate acyltransferase (GPAT) is involved in triacylglycerol (TAG) and phospholipid synthesis, catalyzing the first committed step. In order to further investigate the *in vivo* importance of the dominating mitochondrial variant, GPAT1, a novel *GPAT1*$^{-/-}$ mouse model was generated and studied. Female *GPAT1*$^{-/-}$ mice had reduced body weight-gain and adiposity when fed chow diet compared with littermate wild-type controls. Furthermore, *GPAT1*$^{-/-}$ females on chow diet showed decreased liver TAG content, plasma cholesterol and TAG levels and increased *ex vivo* liver fatty acid oxidation and plasma ketone bodies. However, these beneficial effects were abolished and the glucose tolerance tended to be impaired when *GPAT1*$^{-/-}$ females were fed a long-term high-fat diet (HFD). GPAT1-deficiency was not associated with altered whole body energy expenditure or respiratory exchange ratio. In addition, there were no changes in male *GPAT1*$^{-/-}$ mice fed either diet except for increased plasma ketone bodies on chow diet, indicating a gender-specific phenotype. Thus, GPAT1-deficiency does not protect against HFD-induced obesity, hepatic steatosis or whole body glucose intolerance.

There are two major pathways for TAG synthesis, (1) the monoacylglycerol pathway, which is important following nutrient absorption and (2) the glycerol-3-phosphate pathway involved in *de novo* lipogenesis. The first committed rate-limiting step in the glycerol-3-phosphate pathway is catalyzed by glycerol-3-phosphate acyltransferase (GPAT). Two mitochondrial GPAT isoforms (GPAT1 and GPAT2) [1,2] and two microsomal GPAT variants (GPAT3 and GPAT4) [3–5] have been identified. In most tissues, GPAT1 constitutes less than 10% of total GPAT activity. However, in liver, the activity of GPAT1 constitutes up to 50% of the total activity [6], suggesting that GPAT1 has a major role in liver lipid biosynthesis.

GPAT1 mRNA is upregulated by a high-carbohydrate, fat-free diet and by insulin administration to streptozotocin-diabetic mice [1]. In addition, GPAT1 activity is increased in livers from both diet-induced obese mice and leptin-deficient *ob*/*ob* mice compared with respective lean controls [7]. Thus, GPAT1 seems to act as a sensor/regulator of lipid biosynthesis in response to nutritional and hormonal status. Overexpression of GPAT1 in Chinese hamster ovary cells and primary rat hepatocytes directs incorporation of exogenous fatty acids into TAG and diacylglycerol (DAG) rather than phospholipids [7–9]. Liver-directed overexpression of GPAT1 in mice [10] and rats [11] results in hepatic steatosis with massive accumulation of DAG and TAG in the liver and increased plasma TAG and cholesterol levels. In addition, GPAT1 overexpression increases liver TAG secretion [10] and promotes hepatic insulin resistance [11]. Thus, GPAT1 is upregulated in obesity and insulin resistance and is associated with hepatic lipid accumulation, hypertriglyceridemia and hepatic insulin resistance.

Overexpression of GPAT1 in rat hepatocytes or *in vivo* in mice results in a sharp reduction in fatty acid oxidation [7,9,10]. The opposite may also occur since GPAT1-deficient (*GPAT1*$^{-/-}$) mice have lowered hepatic TAG content [12] and increased levels of β-hydroxybutyrate [13,14], compared with wild-type control mice. Thus, there seems to be a competition for acyl-CoAs between GPAT1 and carnitine palmitoyltransferase 1 (CPT1).

Female *GPAT1*$^{-/-}$ mice have reduced body weight gain and adipose tissue weight [12], while male *GPAT1*$^{-/-}$ mice have similar body weight as controls [12–14]. Conflicting results exist regarding the effect of GPAT1-deficiency on whole body glucose tolerance [13,14]. However, after a short-term HFD, *GPAT1*$^{-/-}$ mice have

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improved hepatic insulin sensitivity compared to wild-type controls [13]. This may be due to reduced liver accumulation of DAG and TAG in *GPAT1*$^{-/-}$ mice [12–14].

Thus, inhibiting GPAT1 may be an attractive way to interfere with the increased TAG accumulation in liver observed in diabetic patients and insulin-resistant individuals [15,16]. In this study, a novel *GPAT1*$^{-/-}$ mouse strain was generated to further investigate the effect of GPAT1-deficiency following long-term HFD treatment on obesity, whole body energy expenditure, glucose tolerance and liver and plasma lipid levels.

Materials and methods

Generation of GPAT1$^{-/-}$ *mice.* A triple LoxP strategy was used to target the *GPAT1* locus by floxing exons 3 and 4 (predicted to introduce a stop codon) in order to generate mice with both standard and conditional knock-out alleles at this locus. The targeting vector was a ~8.4 kb 129/SvJ mouse genomic subclone containing a floxed inverted neomycin phosphotransferase selectable marker cassette inserted into intron 2 and a single LoxP site inserted into intron 4 (Supplemental Fig. IA). After linearization, the targeting construct was electroporated into R1 ES cells (derived from 129/SvJ). PCR screens and Southern blot analyses revealed that 2 out of 505 G418-resistant clones had undergone the desired homologous recombination reaction (Supplemental Fig. IB and C). One of these clones was expanded and injected into C57Bl/6 blastocysts to generate chimeric mice. Chimeric males were crossed with C57Bl/6 females and genotyping of the *agouti* offspring was performed from tail biopsies using a forward primer (F) in intron 2 within the short arm homology 5′-GGTCAGGTGATGTGGCTGTGAT-3′ combined with a reverse neo-specific primer (R1) 5′-GCTGACCGCTTCCTCGTGCTTT-3′. Heterozygous triple LoxP mice were then bred to a constitutive Cre deleter strain of mice in order to generate heterozygous *GPAT*$^{+/-}$ mice, which had undergone Cre-mediated deletion of the intervening region of DNA between the outermost LoxP sites. Such mice were identified using a forward primer (F) in intron 2; 5′-GGTCAGGTGATGTGGCTGTGAT-3′ and a reverse primer (R2) in intron 4; 5′-AACCTTCCTGGCCACATTTA-3′. These primers gave a product of 417 bp for the Cre-recombined (deleted) allele and a 3.38 kb product for the wild-type allele (Supplemental Fig. ID). Heterozygous *GPAT1*$^{+/-}$ mice were then intercrossed to generate homozygous *GPAT1*$^{-/-}$ animals, which proved to be viable.

Diets, body weights and termination. Mice were given either a chow diet containing 12% (energy%) fat, 62% carbohydrates and 26% protein, with a total energy content of 12.6 kJ/g (R3, Lactamin AB, Kimstad, Sweden) or a HFD containing 39.9% fat (mainly saturated), 42.3% carbohydrates and 17% protein, supplemented with 0.15% cholesterol, with a total energy content of 21.4 kJ/g (R638, Lactamin AB). At termination, mice were fasted for 4 h before collection of plasma and organs from isoflurane (Forene, Abbot Scandinavia AB, Sweden) anesthetized mice. All procedures involving animals were conducted in accordance with accepted standards of good animal practice and approved by the Local Ethics Review Committee on Animal experiments (Gothenburg region).

Body composition, indirect calorimetry and glucose tolerance. Body composition was analyzed in anesthetized mice using a Dual Energy X-ray Absorptiometry (DEXA; PIXImus Lunar, GE Medical Systems, Madison, WI). Volume of oxygen consumed (vO_2) and volume of carbon dioxide produced (vCO_2) were measured using an open circuit calorimetry system (Oxymax, Columbus Instruments International Corp., Columbus, OH) as described before [17]. Energy expenditure (kcal/h) was calculated: $(3.815 + 1.232\text{RER}) \times vO_2$, where RER is the respiratory exchange ratio [volume of CO_2 produced per volume of O_2 consumed (both ml/kg/min)] and vO_2 is the volume of O_2 consumed per h per kg mass of the animal. The value of energy expenditure was corrected to individual body weights. Oral glucose tolerance test (OGTT) was performed after a 4 h fast as described before [17].

Plasma and liver lipid levels. Plasma cholesterol and TAG levels were measured with enzymatic colorimetric assays (Roche Diagnostics). β-Hydroxybutyrate levels were measured using an enzymatic kit (Randox Laboratories Ltd., Crumlin, UK). Liver lipids were measured using HPLC as described before [10].

Ex vivo fatty acid oxidation. Palmitoyl-CoA oxidation was measured in liver homogenates from female mice. Livers were homogenized in 19 volumes of ice-cold buffer (250 mM sucrose, 10 mM Tris–HCl, 1 mM EGTA and 1% BSA, pH 7.4). Samples of this homogenate were diluted 2.5-fold in homogenization buffer. Liver homogenate (50 μl) was then incubated with 450 μl reaction mixture (100 mM sucrose, 10 mM Tris–HCl, 5 mM potassium phosphate, 80 mM KCl, 1 mM $MgCl_2$, 2 mM malate, 2 mM ATP, 1 mM DTT, 0.2 mM EDTA, 2 mM L-carnitine, 0.05 mM CoA, 0.2 mM palmitoyl-CoA, 0.3% BSA (fatty acid free) and 0.125 μCi 1-^{14}C-palmitoyl-CoA, pH 7.4). After 60 min of incubation at 25 °C, the reaction was stopped by addition of 0.1 moles ice-cold perchloric acid. $^{14}CO_2$ produced during the 60 min incubation was collected in 100 μl of 1 M NaOH. ^{14}C counts present in the acid soluble fraction were also measured and combined with the $^{14}CO_2$ values to give the total palmitoyl-CoA oxidation rate. The CO_2 contributed with approximately 10% of the total counts (data not shown).

GPAT activity and protein level. Liver samples were homogenized and following excursion of nuclei, 100,000 g pellets were tested for GPAT activity in the presence and absence of 2 mM *N*-ethylmaleimide (NEM) as described previously [7]. GPAT1 expression was determined using Western blot as described before [7].

Histology. Liver tissues were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned and stained with hematoxylin and eosin (H&E).

Statistics. For group comparisons, Student's *t*-test is used. Oxymax-data was analyzed using a two-way ANOVA. Values are shown as means ± standard error of mean (SEM).

Results

A conditional *GPAT1*$^{-/-}$ mouse model was generated as described in Materials and methods. F1 generation mice were backcrossed to C57BL/6 females for three generations before intercrossing. The intercross resulted in normal litter sizes and Mendelian distribution of genotypes (data not shown) and littermate wild-type mice were used as controls throughout this study. Absence of GPAT1 protein in *GPAT1*$^{-/-}$ mice was confirmed using Western blot on mitochondrial protein preparations from mouse liver (Fig. 1A). Feeding wild-type mice a HFD for 14 weeks increased the microsomal GPAT activity by 31% ($P < 0.05$, Student's *t*-test) and tended to increase mitochondrial GPAT activity (23%, $P < 0.11$, Student's *t*-test) in total liver membrane fractions. Mitochondrial GPAT activity was reduced by 85% in *GPAT1*$^{-/-}$ mice fed chow diet and by ~93% when fed a HFD (Fig. 1B), compared to respective wild-type controls. Microsomal GPAT activity was reduced by 50% or 45% in *GPAT1*$^{-/-}$ mice when fed either chow or HFD, respectively compared with wild-type controls (Fig. 1B). Thus, the generated *GPAT1*$^{-/-}$ mice lack GPAT1 protein and have markedly reduced mitochondrial GPAT activity but also reduced microsomal GPAT activity.

Male *GPAT1*$^{-/-}$ mice did not differ in body weight-gain compared to wild-type controls when fed either chow diet or HFD (Fig. 2A). Female *GPAT1*$^{-/-}$ mice gained less weight than wild-type controls when fed chow (Fig. 2B). However, when given HFD, this difference disappeared and tended to be reversed (Fig. 2B). Female *GPAT1*$^{-/-}$ mice fed chow diet showed 27% decreased total body fat mass compared with wild-type controls (Fig. 2C). Conversely, when the mice were fed HFD, no difference was observed between genotypes (Fig. 2C). Male *GPAT1*$^{-/-}$ mice showed no difference in total body fat mass on either diet compared with wild-type controls (Supplemental Fig. IIA). Female *GPAT1*$^{-/-}$ mice fed chow diet had reduced retroperitoneal and ovarian white adipose tissue (WAT) weights compared to wild-type controls (Fig. 2D). However, no difference in WAT weights was observed between genotypes in females fed HFD or in males fed either chow or HFD (Fig. 2D and Supplemental Fig. IIB).

Female *GPAT1*$^{-/-}$ mice fed chow diet had decreased liver TAG levels and intracellular droplets in hepatocytes as demonstrated by biochemical quantification and histological analysis of liver sections, respectively (Fig. 2E). After prolonged HFD, this difference was abolished (Fig. 2F). The liver levels of cholesterol, cholesterol esters, DAG, phosphatidylethanolamine and phosphatidylcholine were unchanged between genotypes in female mice on HFD (data not shown). Female *GPAT1*$^{-/-}$ mice fed chow diet had decreased plasma TAG and cholesterol levels compared with wild-type controls (Table 1), while no change was observed on HFD (Table 1). Plasma TAG levels were not influenced by GPAT1-deficiency in male mice (data not shown). In summary, chow fed female *GPAT1*$^{-/-}$ mice had decreased body weight-gain, adipose tissue mass, liver TAG content and plasma TAG and cholesterol levels but the differences between genotypes disappeared when the mice were fed a HFD for 14 weeks.

We hypothesized that loss of GPAT1 would result in increased fatty acid oxidation due to increased fatty acid substrate availability for CPT1. To test this, liver homogenates were assayed for

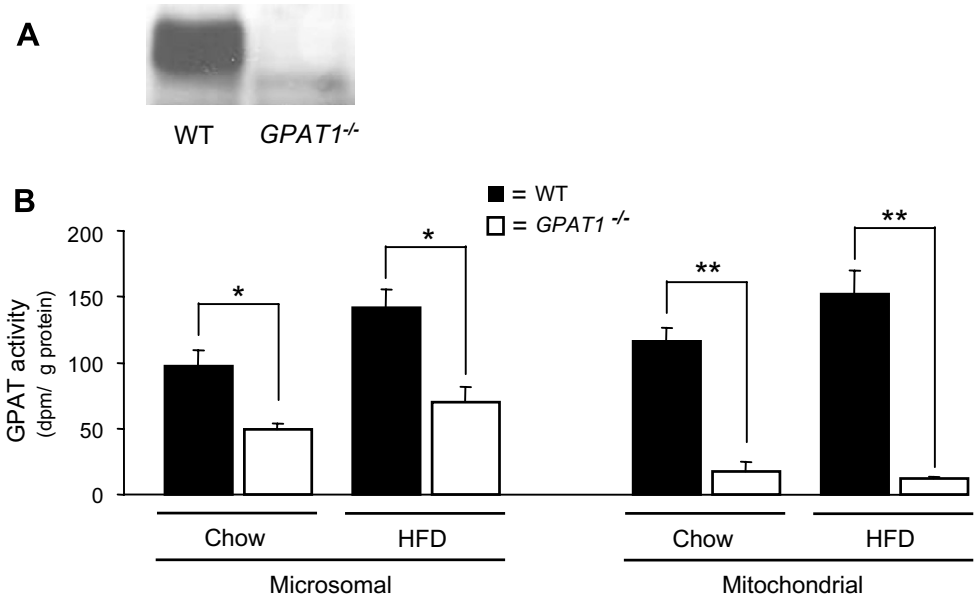

Fig. 1. Verification of the *GPAT1*$^{-/-}$ mouse. (A) GPAT1 protein expression in liver mitochondria determined with Western blot. (B) Microsomal and mitochondrial NEM-sensitive GPAT activity in liver total membrane preparations from 24 weeks old female mice fed chow diet or a high-fat diet for the last 14 weeks. Values are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, Student's *t*-test.

ex vivo fatty acid oxidation. We observed a 15% increase in fatty acid oxidation in female *GPAT1*$^{-/-}$ mice fed chow diet compared with wild-type controls (Fig. 3A). A similar trend was also detected when the mice were fed HFD, although this did not reach statistical significance (Fig. 3A). Female *GPAT1*$^{-/-}$ mice had three-fold increased plasma β-hydroxybutyrate levels, compared with wild-type controls when fed chow diet (Fig. 3B). However, the increase in plasma β-hydroxybutyrate levels was only 59% when the mice were fed HFD (Fig. 3B). Plasma β-hydroxybutyrate levels were to a minor extent also increased in male *GPAT1*$^{-/-}$ mice fed chow diet (47%, $P < 0.05$, Student's *t*-test), but not significantly altered when the mice were fed HFD (Supplemental Fig. IIC). In order to investigate the impact of GPAT1-deficiency on whole body energy expenditure, an open circuit indirect calorimetry system was used. Whole body energy expenditure was unchanged in *GPAT1*$^{-/-}$ mice of either gender compared to wild-type controls (Fig. 3C and Supplemental Fig. IIIA). In addition, respiratory exchange ratio (RER) was unchanged in both female and male *GPAT1*$^{-/-}$ mice compared to wild-type controls (Fig. 3D and Supplemental Fig. IIIB). Thus, the increase in liver fatty acid oxidation capacity and plasma β-hydroxybutyrate levels in *GPAT1*$^{-/-}$ mice fed chow diet was reduced when the mice were fed a HFD. In addition, the increased liver fatty acid oxidation capacity was not associated with changes in whole body energy expenditure or RER.

The glucose tolerance during HFD was determined using oral glucose tolerance tests (OGTT:s). At each time point, female *GPAT1*$^{-/-}$ mice tended to have increased plasma levels of glucose and insulin compared with wild-type controls (Fig. 3E and F), although there was no significant difference in the area under curve (AUC) values (glucose; wt = 100 ± 3.5 vs. *GPAT1*$^{-/-}$ mice 116.1 ± 8.1 (AUC% of wt), insulin; wt = 100 ± 14.1 vs. *GPAT1*$^{-/-}$ mice 144 ± 16.6 (AUC% of wt), $n = 6–8$). However, an increased insulin resistance (IR)-index based on the product of the glucose and insulin OGTT AUC values (wt = 100 ± 3.5 vs. *GPAT1*$^{-/-}$ mice 165.9 ± 18.2, $P < 0.05$, student's *t*-test) was calculated in female *GPAT1*$^{-/-}$ mice compared to wild-type controls. No differences were detected in the OGTT glucose and insulin curves between genotypes in male mice fed HFD (Supplemental Fig. IIIC and D). In addition, no difference in IR-index was calculated in male mice between genotypes (data not shown). Thus, *GPAT1*$^{-/-}$ females but not males display increased IR-index after 12 weeks HFD compared to corresponding wild-type mice.

Discussion

GPAT1 has been shown to be important for TAG biosynthesis in both overexpression and knock-down studies [7–14,18]. However, using a novel *GPAT1*$^{-/-}$ mouse model, we now show that GPAT1-deficiency does not protect against prolonged HFD-induced weight-gain, adiposity, glucose intolerance or liver and plasma lipid accumulation.

Female *GPAT1*$^{-/-}$ mice fed chow diet had reduced body weight-gain, adipose tissue weight and liver and plasma TAG accumulation in accordance with Hammond et al. [12]. However, all these beneficial effects were abolished when the mice were fed HFD for 14 weeks. Only a small proportion of hepatic TAG is suggested to be derived from *de novo* lipogenesis when consuming a HFD [19], although the opposite may occur following a high-carbohydrate diet [20]. Thus, on chow diet, when the majority of calories are derived from carbohydrates, GPAT1-deficiency has a major impact on the hepatic lipid biosynthesis. However, the importance of *de novo* lipogenesis and subsequently GPAT1 activity may be limited when the mice are fed a HFD.

Both female and male *GPAT1*$^{-/-}$ mice had elevated plasma levels of β-hydroxybutyrate, which is used as a plasma marker for hepatic fatty acid oxidation. Similar results have been presented previously in *GPAT1*$^{-/-}$ mice, however no direct measurements of fatty acid oxidation or whole-body energy expenditure were made in these studies [13,14]. We have previously shown that GPAT1 overexpression in rat hepatocytes [7] and liver-directed overexpression of GPAT1 [10] reduces fatty acid oxidation. In further support for this concept we now observed increased *ex vivo* fatty acid oxidation in liver homogenates from *GPAT1*$^{-/-}$ mice. However, this increase in liver fatty acid oxidation was not sufficient to translate

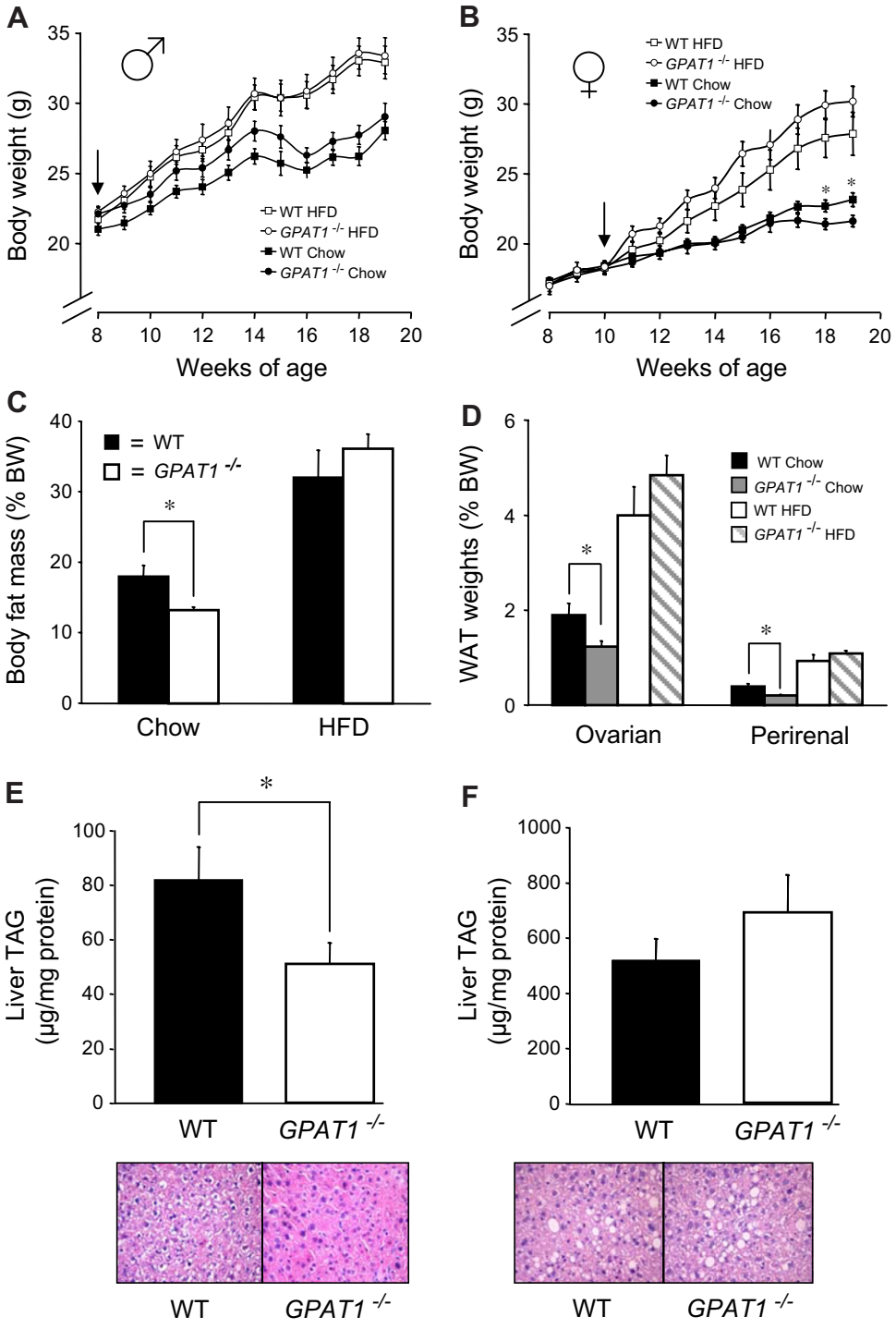

Fig. 2. Body weight gain, adipose tissue mass and weights and liver triacylglycerol (TAG) content. (A) Body weight curves for males fed chow diet or a high-fat diet (HFD) starting at 8 weeks of age (indicated by an arrow, $n = 7–8$). (B) Body weight curves for females fed chow or HFD starting at 10 weeks of age (indicated by an arrow, $n = 7–9$). (C) Quantitative assessment of body fat mass in 21 weeks old females by DEXA ($n = 7–9$). (D) Dissected ovarian and perirenal white adipose tissue (WAT) weights in 24 weeks old females ($n = 7–9$). (E and F) Liver TAG content and representative liver paraffin sections stained with hematoxylin and eosin in female mice fed chow diet (E, $n = 8$) or a HFD for the last 14 weeks (F, $n = 7$-9). Values are means ± SEM. $^{*}P < 0.05$, Student's *t*-test.

Table 1
Plasma levels of triacylglycerol and cholesterol in female *GPAT1*$^{-/-}$ mice and wild-type mice

	Chow diet		High-fat diet	
	Wild-type	*GPAT1*$^{-/-}$	Wild-type	*GPAT1*$^{-/-}$
TAG (mM)	0.59 ± 0.04 ($n = 8$)	0.37 ± 0.03** ($n = 8$)	0.29 ± 0.03 ($n = 9$)	0.24 ± 0.02 ($n = 7$)
Chol (mM)	2.24 ± 0.09 ($n = 8$)	1.92 ± 0.08* ($n = 8$)	3.24 ± 0.23 ($n = 9$)	2.69 ± 0.22 ($n = 7$)

Mice were fed chow or high-fat diet for 14 weeks. Values are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, Student's *t*-test.

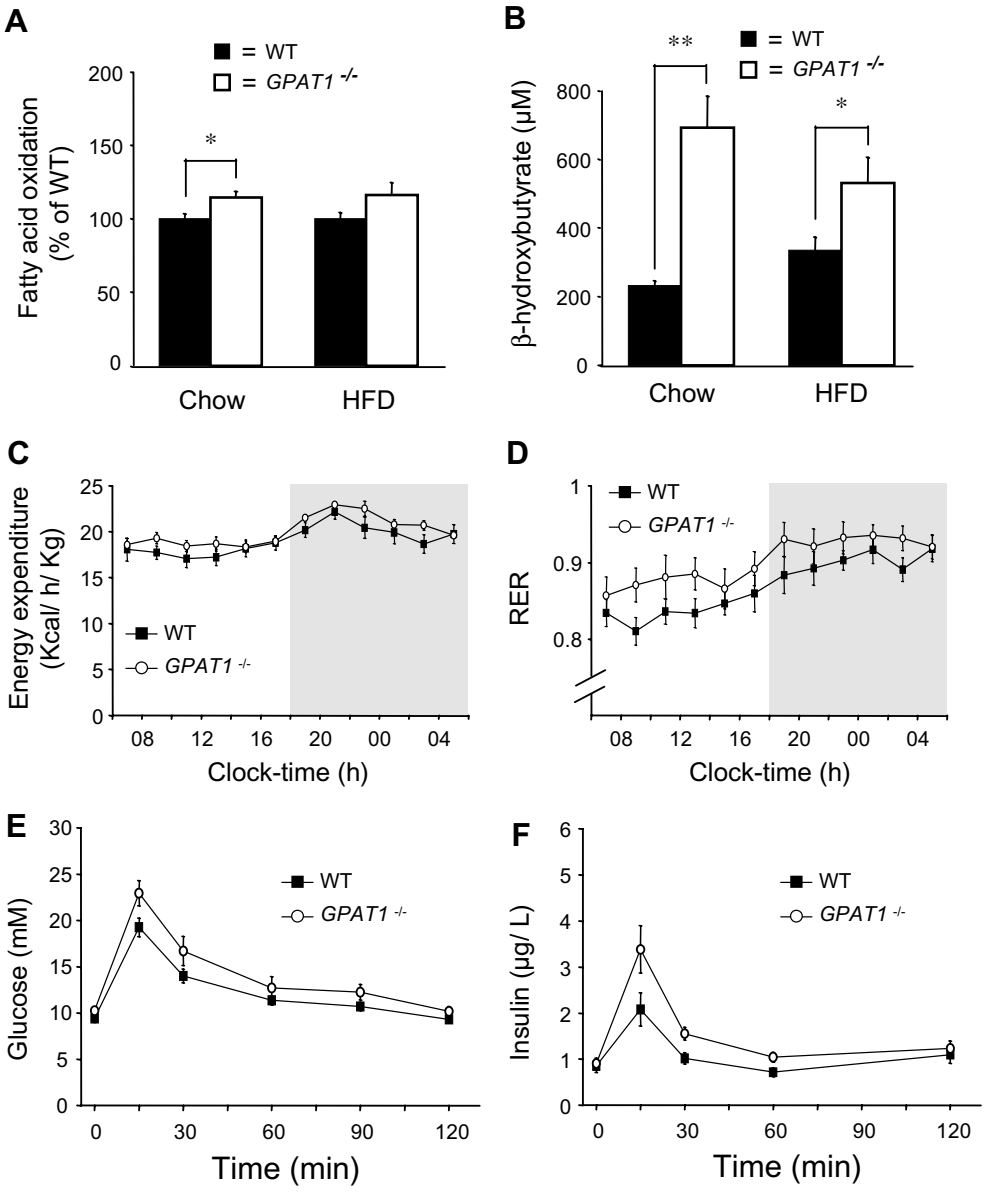

Fig. 3. Liver fatty acid oxidation, energy expenditure and oral glucose tolerance. (A) Liver fatty acid oxidation assessed in freshly isolated liver homogenate in 24 weeks old females ($n = 6–9$) using ^{14}C-palmitoyl-CoA as tracer as described in Materials and methods. (B) Plasma levels of β-hydroxybutyrate in 24 weeks old females ($n = 7–9$). (C) Energy expenditure and (D) respiratory exchange ratio (RER) measured with an open circuit calorimetry system in 20-week-old females on a chow diet (dark period shaded, $n = 6–7$). (E and F) Oral glucose tolerance test in 22 weeks old females fed a high-fat diet for the last 12 weeks. Plasma glucose (E) and insulin (F) were measured before and 15, 30, 60, 90 (not insulin) and 120 min after an oral glucose load (2 g/kg). Values are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, Student's *t*-test.

into increased whole body energy expenditure or changed respiratory exchange ratio, indicating that GPAT1-deficiency does not have a major impact on whole body energy combustion.

Our result of increased IR-index in female *GPAT1*$^{-/-}$ mice after 12 weeks of HFD is in line with the results from male mice fed a HFD (from coconut oil) for 16 weeks [14]. In contrast, feeding *GPAT1*$^{-/-}$ mice a safflower oil diet resulted in increased glucose tolerance and improved hepatic insulin sensitivity compared to wild-type mice [13]. Thus, the effects of GPAT1-deficiency on glucose tolerance are highly dependent upon gender, availability and composition of dietary lipids and also the carbohydrate content of the diet.

In line with our previous data, feeding mice a HFD increased microsomal and tended to increase mitochondrial GPAT activity [7]. A residual activity of mitochondrial GPAT of about 10% was detected in *GPAT1*$^{-/-}$ mice, as shown previously [12]. This may be due to incomplete NEM inactivation of microsomal GPAT, which is present in total membrane fractions. The reasons for the decreased microsomal GPAT activity in *GPAT1*$^{-/-}$ mice are unclear and contrasts the data from the *GPAT1*$^{-/-}$ mice produced by Hammond et al. [12]. However, the lack of beneficial metabolic effects in *GPAT1*$^{-/-}$ mice following a long-term HFD described in this paper, is at least not due to compensatory upregulation of microsomal GPAT activity.

Knockdown and overexpression studies confirm the important regulatory role of GPAT1 in liver TAG biosynthesis. However, this study shows that GPAT1 is not an essential enzyme in hepatic TAG synthesis and that GPAT1-deficiency does not protect against high-fat diet-induced obesity or glucose intolerance and does not increase whole body energy expenditure. Thus, beneficial effects

of GPAT1 inhibition as treatment for obesity, fatty liver and insulin resistance may be questioned.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.02.156.

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