Lack of FFAR1/GPR40 Does Not Protect Mice From High-Fat Diet–Induced Metabolic Disease

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OBJECTIVE—FFAR1/GPR40 is a G-protein–coupled receptor expressed predominantly in pancreatic islets mediating free fatty acid–induced insulin secretion. However, the physiological role of FFAR1 remains controversial. It was previously reported that FFAR1 knockout ($Ffar1^{-/-}$) mice were resistant to high-fat diet–induced hyperinuslinemia, hyperglycemia, hypertriglyceridemia, and hepatic steatosis. A more recent report suggested that although FFAR1 was necessary for fatty acid–induced insulin secretion in vivo, deletion of FFAR1 did not protect pancreatic islets against fatty acid–induced islet dysfunction. This study is designed to investigate FFAR1 function in vivo using a third line of independently generated $Ffar1^{-/-}$ mice in the C57BL/6 background.

RESEARCH DESIGN AND METHODS—We used CL-316,243, a β3 adrenergic receptor agonist, to acutely elevate blood free fatty acids and to study its effect on insulin secretion in vivo. $Ffar1^{+/+}$ (wild-type) and $Ffar1^{-/-}$ (knockout) mice were placed on two distinct high-fat diets to study their response to diet-induced obesity.

RESULTS—Insulin secretion was reduced by ~50% in $Ffar1^{-/-}$ mice, confirming that FFAR1 contributes significantly to fatty acid stimulation of insulin secretion in vivo. However, $Ffar1^{+/+}$ and $Ffar1^{-/-}$ mice had similar weight, adiposity, and hyperinsulinemia on high-fat diets, and $Ffar1^{-/-}$ mice showed no improvement in glucose or insulin tolerance tests. In addition, high-fat diet induced comparable levels of lipid accumulation in livers of $Ffar1^{+/+}$ and $Ffar1^{-/-}$ mice.

CONCLUSIONS—FFAR1 is required for normal insulin secretion in response to fatty acids; however, $Ffar1^{-/-}$ mice are not protected from high-fat diet–induced insulin resistance or hepatic steatosis. *Diabetes* **57:2999–3006, 2008**

Fatty acids are involved in a diverse array of physiological functions in a variety of tissues. Many of the effects of fatty acids are thought to be mediated by intracellular metabolites of long-chain acyl-CoA esters. Evidence has accumulated over the last few years, however, that at least some of the activities attributed to fatty acids are mediated through their interaction with a number of G-protein–coupled receptors

designated FFAR1 (GPR40), FFAR2 (GPR43), FFAR3 (GPR41), GPR120, and GPR84. FFAR2 and FFAR3 are activated by short-chain fatty acids, whereas FFAR1, GPR84, and GPR120 are activated by medium- to long-chain fatty acids (1). Among these receptors, FFAR1 and GPR120 have drawn attention for their potential as therapeutic targets for metabolic disease (2–4).

Interest in FFAR1 intensified in 2005 when Steneberg et al. (5) reported that mice lacking FFAR1 were resistant to a number of the effects of a high-fat diet, including hyperinuslinemia, hyperglycemia, hypertriglyceridemia, and hepatic steatosis. Conversely, Steneberg et al. also reported that transgenic mice overexpressing FFAR1 in pancreatic islets developed diabetes, suggesting that sustained signaling through FFAR1 could be detrimental. Although it is known that fatty acids are important for insulin secretion in normal individuals, it is also widely recognized that chronically elevated fatty acids contribute to pancreatic dysfunction (6). In this regard, Steneberg et al. observed that insulin secretion from isolated $Ffar1^{-/-}$ islets was not impaired by 48-h exposure to palmitic acid (5). They concluded that $Ffar1^{-/-}$ islets are protected from the long-term negative effect of fatty acids and suggested that FFAR1 antagonists might be used to prevent and treat obesity-associated type 2 diabetes. Recently, Latour et al. (7) reported the characterizations of a second $Ffar1^{-/-}$ line. They showed that $Ffar1^{-/-}$ mice had a ~50% reduction in acute insulin secretion in response to a bolus of injected Intralipid. Although Latour et al. did not report on the effects of high-fat feeding in their $Ffar1^{-/-}$ mice, they observed that male knockout mice on a chow diet developed significant (albeit slight) glucose intolerance. They also showed that isolated islets from $Ffar1^{-/-}$ mice were still subject to the impairment of the insulin response after chronic exposure to fatty acids (7). Except for the role of FFAR1 in mediating insulin secretion, these findings stand in contrast to the report of Steneberg et al. (5).

Here, we report metabolic phenotypes on a third, independent line of $Ffar1^{-/-}$ mice generated in the C57BL/6Ncrl (B6) background. We found that FFAR1 contributes to ~50% of fatty acid–mediated insulin release in vivo, consistent with the previous report of Latour et al. (7). In contrast to the previous observations by Steneberg et al. (5), however, our $Ffar1^{-/-}$ mice are not protected from the negative effects of high-fat diet. $Ffar1^{-/-}$ mice on a high-fat diet became obese, developed insulin resistance, and accumulated liver lipids to a similar extent when compared with $Ffar1^{+/+}$ littermates. Taken together, our data confirm that FFAR1 is important for fatty acid–mediated insulin secretion, but call into question its role in mediating the toxic effects of free fatty acids on metabolic diseases.
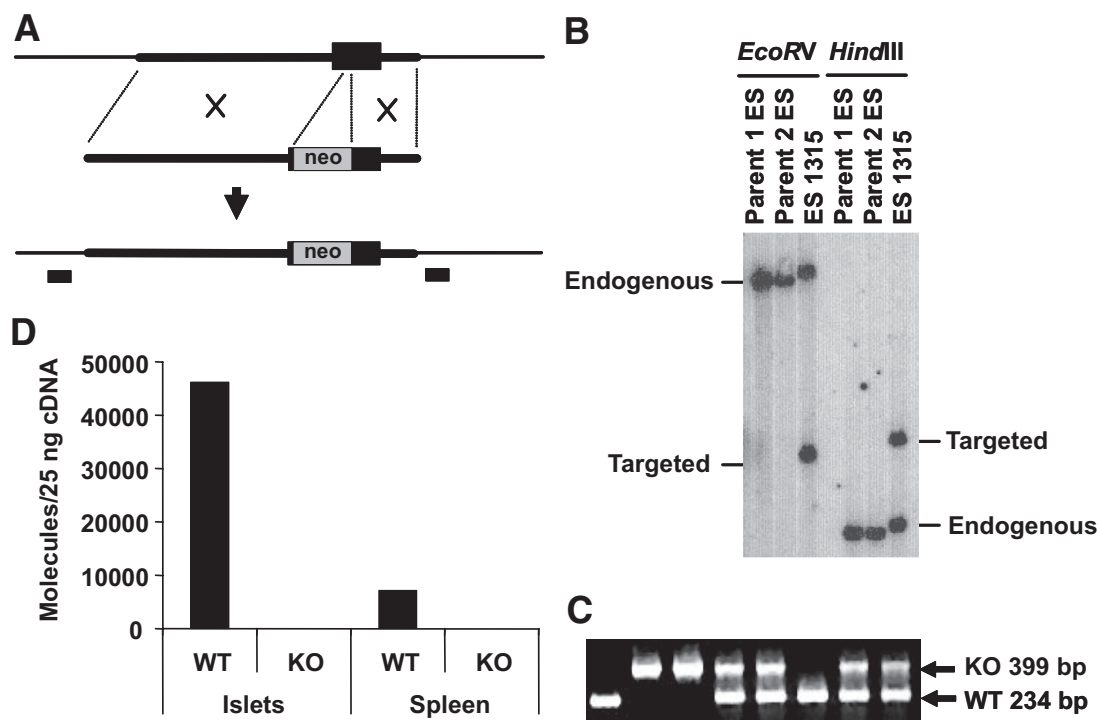
FIG. 1. Generation of *Ffar1*$^{-/-}$ mice. *A*: Schematic view of the regions depleted in *Ffar1* gene. A 152-bp fragment corresponding to position 143–294 of the open reading frame of the *Ffar1* gene (NM_194057) was replaced by a *neo* cassette. The two black bars below the *bottom panel* represent the positions of the 5′ and 3′ probes used for Southern blotting. *B*: Southern blotting of ES cell clones. Shown are DNA samples from two nontargeted ES clones and a targeted clone (ES 1315) digested by *Eco*RV and probed with a 5′ probe and by *Hin*dIII and probed with a 3′ probe, respectively. *C*: Representative PCR genotyping of DNA samples from tail clips of *Ffar1*$^{+/+}$ (WT) and *Ffar1*$^{-/-}$ (KO) mice. The WT allele is 234 bp and the KO allele is 399 bp in size. *D*: Expression of *Ffar1* mRNA in pancreatic islets and spleen of *Ffar1*$^{+/+}$ (WT) and *Ffar1*$^{-/-}$ (KO) mice accessed by real-time quantitative PCR.

RESEARCH DESIGN AND METHODS

Generation of *Ffar1*$^{-/-}$ mice in C57BL/6NCrl (B6) background. *Ffar1*$^{-/-}$ mice were custom-generated by DeltaGen (Palo Alto, CA). Briefly, a targeting vector was designed to delete a 152-bp fragment corresponding to position 143–294 of the open reading frame of the *Ffar1* gene (NM_194057). This segment was replaced with a Neomycin (*neo*) cassette. The vector was electroporated into 129/OlaHsd-derived embryo stem (ES) cells, and G418-resistant ES cell colonies were then picked and expanded for DNA analysis. The correctly targeted ES cells were injected into blastocysts to generate chimeric mice that were then mated to B6 females. *Ffar1* heterozygous (*Ffar1*$^{+/-}$) offspring were identified by a PCR-based screening strategy. The PCR primers were designed to detect both wild-type (234-bp) and knockout (399-bp) alleles. Oligonucleotide sequences were as follows: *Ffar1* upstream of the deleted region forward, 5′-cccagcttggtctacactctccatc-3′; *Ffar1* downstream of the deleted region reverse, 5′-gatggcttggtacccgaaggggaag-3′; and *neo* forward, 5′-gggtgggattagataaatgcctgctct-3′. *Ffar1*$^{+/-}$ mice were then interbred to generate *Ffar1*$^{-/-}$ mice. *Ffar1*$^{-/-}$ mice were backcrossed to C57BL/6NCrl mice for >10 generations to generate mice in a B6 background.

In vivo insulin secretion in response to acutely elevated free fatty acids. Nonfasted *Ffar1*$^{+/+}$ and *Ffar1*$^{-/-}$ mice, age and sex matched, received intraperitoneal injection of either saline or the β3-agonist CL-316,243 (Sigma-Aldrich) at 1 mg/kg (8). Fifteen minutes after injection, ~700 μl blood was collected from each mouse by cardiac puncture immediately after CO_2 asphyxiation. To preserve glucagon-like peptide 1 (GLP-1), DPP-IV inhibitor (Linco) was added to precooled blood collection tubes (BD Microtainer 365974; Becton Dickinson, Franklin Lakes, NJ) and plasma isolated by centrifugation at 5,000 rpm for 10 min while maintaining a temperature of 4°C. Aliquots of plasma samples were stored at −80°C until analysis. Plasma levels of insulin and GLP-1 (7-36)amide were estimated using kits from Meso Scale Discovery (Gaithersburg, MD). Leptin, amylin, and glucagon levels were measured using the Linco*plex* Luminex kits (Linco Research, St. Charles, MO). Glucose, free fatty acids, and triglycerides were measured using commercial kits from Wako Diagnostics (Richmond, VA).

High-fat diet–feeding studies. All mice under study were weaned onto Lab Rodent Diet 20 (4.5% fat; PMI Nutrition International) and, unless specified, were group housed in polycarbonate cages in a specific pathogen-free environment on a 12-h light/dark cycle at a temperature of 22°C. In one study, mice at N6 generation of backcrossing into B6 background were fed for 8 weeks with a Surwit diabetogenic diet (58% of calories from lard) or a semipurified low-fat diet (D12450B; 10% of calories from fat; Research Diets, New Brunswick, NJ) ad libitum. In a separate study, mice at ≥N10 of backcrossing into B6 background were fed with a semipurified diet with 60% calories from fat (Research Diets). Body composition was assessed by nuclear magnet resonance spectroscopy. Mice were killed by CO_2 asphyxiation for blood and tissue collection after 4 h fasting. Plasma glucose, fatty acid, and triglyceride levels were measured as described above. Liver neutral lipids (cholesterol ester, free cholesterol, and triglyceride) were analyzed by high-performance liquid chromatography as previously described (9). All studies were conducted in an American Association for Laboratory Animal Care–accredited facility, according to protocols approved by the Schering-Plough Research Institute Animal Care and Use Committee.

Glucose and insulin tolerance test. Blood was collected after 16 h of fasting, and basal glucose levels were measured using a glucose oxidase method (Glucometer Elite; Bayer, Elkhart, IN). After this measure, glucose (3 g/kg body wt) or insulin (0.75 mU/g body wt) was administered by intraperitoneal injection, and blood was collected from the tail vein at 20, 40, 60, 90, and 120 min after dose for glucose determination.

Data analysis. Data are reported as means ± SE. Statistical analyses were performed with GraphPad Prism (version 4.00; GraphPad Software) using unpaired Student's *t* tests, one-way ANOVA, or two-way ANOVA, depending on data structures. Specific methods used are indicated in the text. *P* values <0.05 are considered statistically significant.

RESULTS

To generate *Ffar1*$^{-/-}$ mice, a segment of the gene encoding part of the second transmembrane domain, all of the first extracellular domain, and part of the third transmembrane domain of the FFAR1 protein was targeted by homologous recombination (Fig. 1*A*). Targeted ES cells were first screened by PCR, and positive clones were identified by Southern blotting using probes that hybridize outside of and adjacent to the targeting construct arms. *Ffar1*$^{-/-}$ mice were derived from a positive ES clone (ES 1315) (Fig. 1*B*). Examples of PCR genotypes from individual *Ffar1*$^{+/+}$ (wild-type), *Ffar1*$^{+/-}$ (Het), and *Ffar1*$^{-/-}$
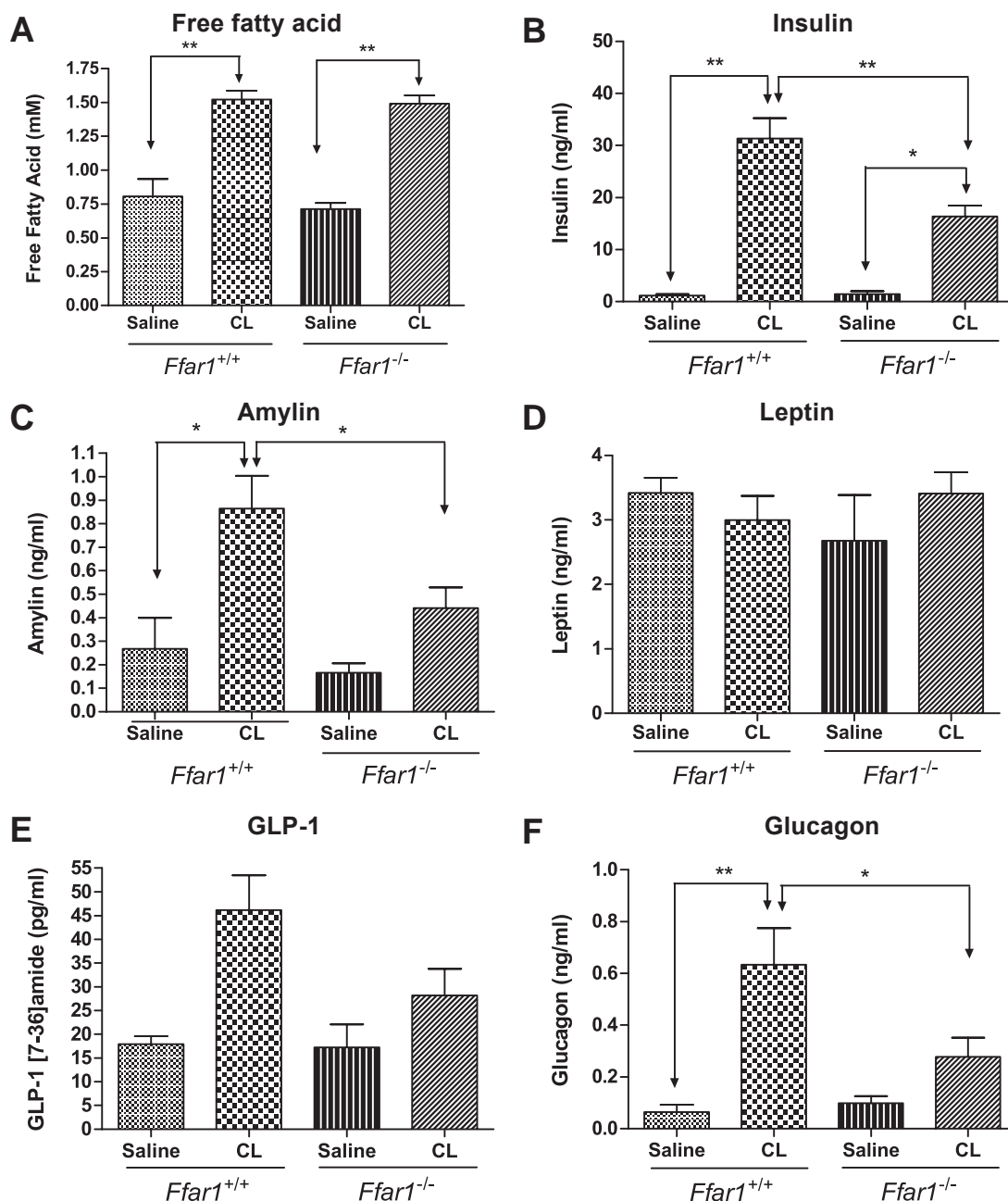
FIG. 2. Plasma parameters of *Ffar1*^{+/+} and *Ffar1*^{−/−} mice in response to β3 agonist CL-316,243 (CL) injections. Mice received intraperitoneal injection of either saline or CL-316,243. The four treatment groups are: *Ffar1*^{+/+} mice receiving saline, *Ffar1*^{+/+} mice receiving CL-316,243, *Ffar1*^{−/−} mice receiving saline, and *Ffar1*^{−/−} mice receiving CL-316,243. *n* = 5–8 mice per group. *A*: Free fatty acids. *B*: Insulin. *C*: Amylin. *D*: Leptin. *E*: GLP-1 (7-36)amide. *F*: Glucagon. The data are analyzed by one-way ANOVA followed by *t* tests with Bonferroni correction. *$P < 0.05$; **$P < 0.01$.

(knockout) mice are shown in Fig. 1*C*. As assessed by real-time quantitative RT-PCR, *Ffar1*^{−/−} mice lack *Ffar1* mRNA expression in pancreatic islets or in spleen (Fig. 1*D*). *Ffar1*^{−/−} mice were born at the expected 1:2:1 Mendelian ratio and appeared healthy and fertile, and no abnormalities were detected on gross examination. Under chow-feeding conditions, we observed no overt difference in metabolic phenotypes between *Ffar1*^{+/+} and *Ffar1*^{−/−} mice.

Fatty acid–stimulated insulin secretion was attenuated in *Ffar1*^{−/−} mice in vivo. To examine the role of FFAR1 in acute fatty acid–stimulated insulin release, we used a selective β3-adrenergic receptor agonist CL-316,243, which promotes lypolysis in rodent white

adipose tissue (10). When administrated by intraperitoneal injection, CL-316,243 increases plasma free fatty acid levels within 5 min, which in turn rapidly increases plasma insulin levels (8). We injected saline and CL-316,243 (1 mg/kg) into *Ffar1*^{+/+} and *Ffar1*^{−/−} mice, respectively, and collected blood samples to measure fatty acid and insulin levels. We used one-way ANOVA to assess the changes among the treatment groups. To test the differences between pairs of groups, we used *t* tests with Bonferroni corrections to adjust for multiple comparisons. As shown in Fig. 2, plasma fatty acid levels increased by approximately twofold in both *Ffar1*^{+/+} and *Ffar1*^{−/−} mice in response to CL-316,243 injection ($P < 0.01$). There was no difference between *Ffar1*^{+/+} and *Ffar1*^{−/−} mice in the
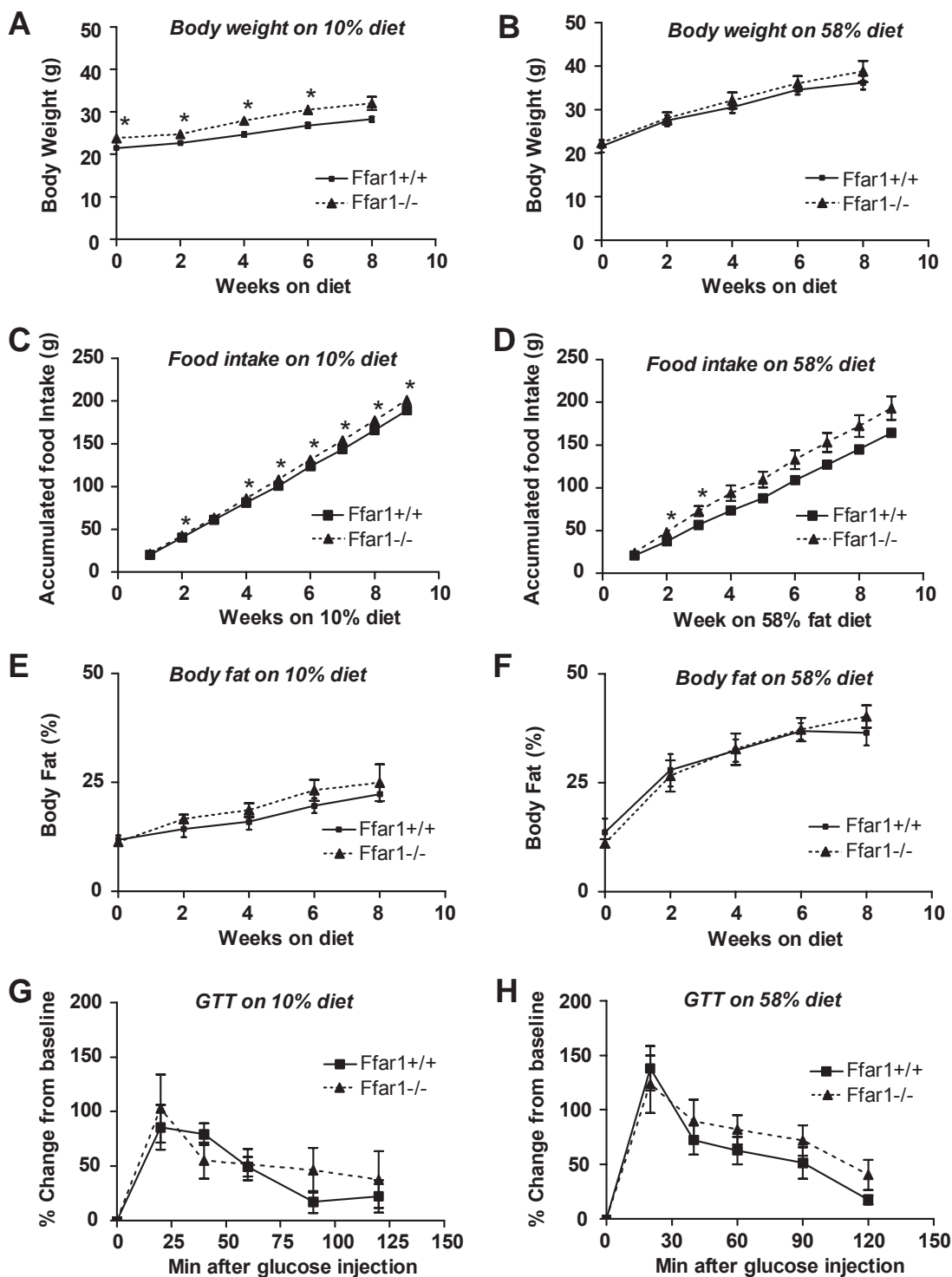
FIG. 3. Metabolic characterizations of *Ffar1*^{+/+} and *Ffar1*^{−/−} mice after 8 weeks on a low-fat diet and high-fat diet. *Ffar1*^{+/+} and *Ffar1*^{−/−} mice at N6 generation of backcrossing into B6 background were fed for 8 weeks on a Surwit diabetogenic diet (58% of calories from lard) or a semipurified low-fat diet (10% of calories from fat) ad libitum. *n* = 5 mice per group. Body weight and body fat were monitored biweekly. Food intake was measured weekly. Glucose tolerance tests (GTT) were performed before mice were killed for blood samples and tissue collection. *A*: Body weight of low-fat diet–fed mice. *B*: Body weight of high-fat diet–fed mice. *C*: Accumulated food intake of low-fat diet–fed mice. *D*: Accumulated food intake of high-fat diet–fed mice. *E*: Body fat of low-fat diet–fed mice. *F*: Body fat of low-fat diet–fed mice. *G*: Glucose tolerance test of low-fat diet–fed mice. *H*: Glucose tolerance test of low-fat diet–fed mice. Data are presented as means ± SE. *$P < 0.05$, *t* test comparing *Ffar1*^{+/+} and *Ffar1*^{−/−} mice at each time point.

CL-316,243–treated groups ($P = 0.77$, pairwise, two-tailed *t* test), indicating that β-adrenergic receptor–mediated lipolysis in adipose tissue was not impaired in *Ffar1*^{−/−} mice (Fig. 2*A*). In *Ffar1*^{+/+} mice, this increase of free fatty acid was accompanied by a ~20-fold increase in plasma insulin level (Fig. 2*B*). In the *Ffar1*^{−/−} mice, insulin levels

were elevated about one-half as much as in the *Ffar1*^{+/+} mice despite a similar increase in fatty acids (Fig. 2*B*; $P < 0.01$, *Ffar1*^{+/+} CL-316,243 vs. *Ffar1*^{−/−} CL-316,243, *t* test with Bonferroni correction). Similar results were obtained in two other independent experiments (data not shown). The data suggest that FFAR1 is responsible for ~50% of

TABLE 1
Plasma and liver parameters of $Ffar1^{+/+}$ and $Ffar1^{-/-}$ mice after 8 weeks on 10% low-fat diet and 58% high-fat diet

| | Low-fat diet | | High-fat diet | | P value | |
	$Ffar1^{+/+}$	$Ffar1^{-/-}$	$Ffar1^{+/+}$	$Ffar1^{-/-}$	Diet	Genotype
n	5	5	5	5		
Plasma glucose (mg/dl)	227 ± 18	236 ± 6	236 ± 16	230 ± 17	NS	NS
Plasma insulin (ng/ml)	0.79 ± 0.18	1.76 ± 0.52	3.54 ± 1.13	3.78 ± 1.13	0.02	NS
Plasma total cholesterol (mg/dl)	108.6 ± 5.4	124.8 ± 7.6	138.5 ± 6.6	139.1 ± 9.1	<0.01	NS
Plasma triglyceride (mg/dl)	68.6 ± 4.5	48.1 ± 6.8	63.4 ± 5.8	55.6 ± 5.2	NS	0.02
Liver cholesterol ester (mg/g tissue)	6.37 ± 0.63	8.34 ± 0.79	5.65 ± 0.43	6.06 ± 0.21	0.02	0.05
Liver triglyceride (mg/g tissue)	62.2 ± 9.8	122 ± 20	226 ± 47	301 ± 60	NS	<0.01
Liver free cholesterol (mg/g tissue)	1.92 ± 0.10	1.64 ± 0.19	1.34 ± 0.13	1.71 ± 0.11	NS	NS
Liver weight (g)	1.11 ± 0.07	1.54 ± 0.06	1.21 ± 0.06	1.33 ± 0.11	NS	0.02
Liver cholesterol ester (mg/liver)	7.09 ± 0.87	12.95 ± 1.61	6.74 ± 0.36	8.10 ± 0.86	0.02	<0.01
Liver triglyceride (mg/liver)	71.3 ± 15.9	193 ± 39	281 ± 72	421 ± 104	<0.01	NS
Liver free cholesterol (mg/liver)	2.10 ± 0.02	2.51 ± 0.25	1.59 ± 0.10	2.23 ± 0.14	0.02	<0.01

Data are means ± SE. The P values are generated by two-way ANOVA testing diet and genotype effects.

fatty acid–stimulated insulin secretion. Amylin release was attenuated in $Ffar1^{-/-}$ mice as well, following the same pattern as that of insulin (Fig. 2C). The ~10-fold increase of insulin in $Ffar1^{-/-}$ mice in response to CL-316,243 could conceivably be due to a direct stimulation of β3-adrenergic receptor–mediated nerve signaling in the pancreatic islets, or it could be mediated indirectly through other adipose or gut-derived hormones, such as leptin or GLP-1. We examined whether leptin or GLP-1 release was affected by CL-316,243. Plasma leptin levels were unaffected (Fig. 2D, $P = 0.61$, ANOVA). For GLP-1, there was a significant difference among the treatment groups as assessed by ANOVA ($P = 0.046$); however, t tests failed to reach statistical significance after adjusting for multiple comparisons. Nevertheless, on pairwise comparison, GLP-1 levels increased by approximately twofold in CL-316,243–treated $Ffar1^{+/+}$ mice compared with saline-treated mice (Fig. 2E; $P = 0.02$, $Ffar1^{+/+}$ CL-316,243 vs. $Ffar1^{+/+}$ saline, one-tailed t test). In the CL-316,243–treated groups, $Ffar1^{-/-}$ mice had lower GLP-1 levels when compared with the $Ffar1^{+/+}$ mice ($P = 0.04$, $Ffar1^{+/+}$ CL-316,243 vs. $Ffar1^{-/-}$ CL-316,243, one-tailed t test). Finally, because it was reported that FFAR1 is expressed and active in pancreatic α-cells (11), we tested the effect of CL-316,243 treatment on glucagon secretion. As shown in Fig. 2F, glucagon levels increased on CL-316,243 treatment ($P < 0.05$, t test with Bonferroni correction), and among the CL-316,243–treated groups, $Ffar1^{-/-}$ mice had lower glucagon levels than the $Ffar1^{+/+}$ mice (Fig. 2F; $P < 0.05$, t test with Bonferroni correction).

$Ffar1^{-/-}$ mice were not protected from high-fat diet–induced metabolic complications. In light of the previous report by Steneberg et al. (5), we asked whether our $Ffar1^{-/-}$ mice are protected from developing insulin resistance under metabolic stress. We first carried out a pilot study using mice on a mixed B6/129 background and a modest high-fat diet (45% calories from fat; Research Diets). We failed to observe any difference in obesity-associated phenotypes between $Ffar1^{+/+}$ and $Ffar1^{-/-}$ mice (data not shown). To test the possibility that different high-fat diet formulas might have led to a different phenotype than that observed by Steneberg et al. (5), we obtained the same diet they used (Surwit Diabetogenic Diet, 58% kcal Lard Diet; custom-made through Research Diets) and an appropriate control semipurified low-fat diet (D12450B; 10% of calories from fat; Research Diets). We

placed five mice of each genotype (total 20 mice; 7–8 weeks old) on either a high-fat or low-fat diet for 8 weeks. The $Ffar1^{-/-}$ mice on the 10% low-fat diet were heavier than the $Ffar1^{+/+}$ mice on the same diet (Fig. 3A), whereas the mice selected for 58% high-fat diet did not show a significant difference (Fig. 3B). $Ffar1^{-/-}$ mice had slightly but significantly higher accumulated food intake than the $Ffar1^{+/+}$ mice on a low-fat diet (Fig. 3C). On a high-fat diet, $Ffar1^{-/-}$ mice also showed more accumulated food intake at weeks 2 and 3 (Fig. 3D); however, for the later time points, the differences did not reach statistical significance (P values by t tests were between 0.05 and 0.10 for each of those time points) (Fig. 3D). Throughout the time course, the body fat percentages were comparable between $Ffar1^{+/+}$ and $Ffar1^{-/-}$ mice (Fig. 3E and F). Glucose tolerance and insulin tolerance tests were performed after the mice were put on a diet for 8 weeks. In contrast to what was reported by Steneberg et al. (5), $Ffar1^{-/-}$ mice did not exhibit improved glucose tolerance when fed either the low-fat diet (Fig. 3G) or high-fat diet (Fig. 3H).

At the end of the diet study, we measured plasma metabolic parameters using samples collected from terminal bleeds (Table 1). We used two-way ANOVA to analyze the differences attributed to diet and genotype effects. If there was a significant genotypic effect, we then used pairwise t tests to examine whether the difference was attributed to a low-fat diet or high-fat diet. We did not observe a diet or a genotypic effect on plasma glucose. The glucose levels were high in all the groups, likely as a result of postprandial terminal bleeding. There was a significant diet effect, but not a genotypic effect, on insulin (Table 1; $P = 0.02$). Both $Ffar1^{+/+}$ and $Ffar1^{-/-}$ mice had comparable insulin levels on the high-fat diet, and the insulin levels between the $Ffar1^{+/+}$ and $Ffar1^{-/-}$ mice on the low-fat diet were not significantly different ($P = 0.11$, pairwise t test). The data indicate that $Ffar1^{-/-}$ mice were not protected from high-fat diet–induced hyperinsulinemia. Plasma cholesterol levels were also higher in high-fat diet–fed mice, and there was no genotypic effect. Interestingly, there was a genotypic effect on plasma triglycerides ($P = 0.02$, two-way ANOVA). The effect was attributed to the fact that the $Ffar1^{-/-}$ mice had lower plasma triglyceride levels on the low-fat diet ($P = 0.03$, t test), but not on the high-fat diet ($P = 0.31$, t test). Conversely, the liver triglyceride levels were higher in the
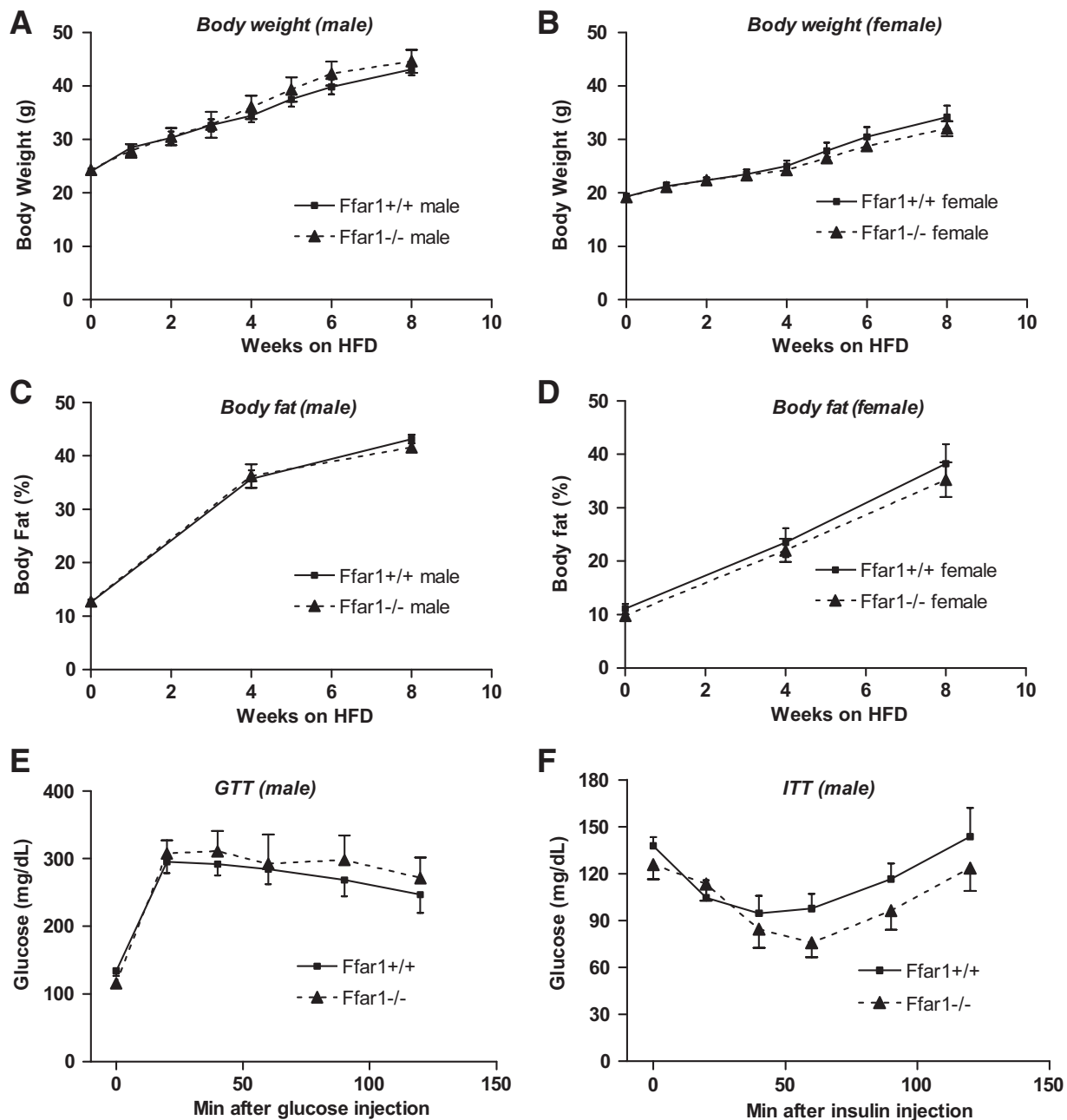
FIG. 4. Metabolic characterizations of *Ffar1*$^{+/+}$ and *Ffar1*$^{-/-}$ mice after 10 weeks on 60% high-fat diet (HFD). *Ffar1*$^{+/+}$ and *Ffar1*$^{-/-}$ mice after 10 generations of backcrossing into B6 background were fed for 10 weeks on semipurified high-fat diet (60% calories from fat). $n = 11$, *Ffar1*$^{+/+}$ male; $n = 9$, *Ffar1*$^{-/-}$ male; $n = 10$, *Ffar1*$^{+/+}$ female; $n = 10$, *Ffar1*$^{-/-}$ female. Body weight was monitored weekly, and body fat was assessed every 4 weeks. Glucose tolerance tests (GTT) and insulin tolerance tests (ITT) were performed at the end of the study. *A*: Body weight of male mice. *B*: Body weight of female mice. *C*: Body fat of male mice. *D*: Body fat of female mice. *E*: GTT of male mice. *F*: ITT of male mice. Data are presented as means ± SE.

Ffar1$^{-/-}$ mice on a low-fat diet ($P = 0.03$, t test). High-fat diet increased liver triglyceride levels in both *Ffar1*$^{+/+}$ and *Ffar1*$^{-/-}$ mice to a comparable level, so it is apparent that *Ffar1*$^{-/-}$ mice were not protected from high-fat diet–induced hepatic steatosis.

Because we were unable to reproduce the phenotype of the *Ffar1*$^{-/-}$ mice using the 58% Surwit lard diet, we carried out a third high-fat diet study using a semipurified 60% high-fat diet from Research Diets. In addition, to eliminate contribution of a mixed genetic background, we further backcrossed our mice into a B6 background for more than 10 generations. Forty mice (*Ffar1*$^{+/+}$ male, $n = 11$; *Ffar1*$^{-/-}$ male, $n = 9$; *Ffar1*$^{+/+}$ female, $n = 10$;

Ffar1$^{-/-}$ female, $n = 10$) at 6–8 weeks of age were placed on the 60% high-fat diet for 10 weeks. Metabolic characterization was performed before and after the high-fat diet treatment. As shown in Fig. 4, mice of both genotypes had similar weight gain during the course of high-fat feeding (Fig. 4*A* and *B*), and percentage of body fat did not differ between *Ffar1*$^{+/+}$ and *Ffar1*$^{-/-}$ mice (Fig. 4*C* and *D*). Again, *Ffar1*$^{-/-}$ mice did not show improvement on the glucose tolerance test (Fig. 4*E*). No significant differences were observed between the genotypes at any of the time points in the insulin tolerance test (Fig. 4*F*), and the areas under the curves did not differ significantly ($13,449 \pm 881$ vs. $11,845 \pm 1,170$, $P = 0.28$, t test). We also measured liver

TABLE 2
Liver lipids of $Ffar1^{+/+}$ and $Ffar1^{-/-}$ mice after 10 weeks on a 60% high-fat diet

	Male		Female		P value	
	$Ffar1^{+/+}$	$Ffar1^{-/-}$	$Ffar1^{+/+}$	$Ffar1^{-/-}$	Sex	Genotype
n	11	9	10	10		
Liver cholesterol ester (mg/g tissue)	4.42 ± 0.80	4.88 ± 1.03	4.48 ± 0.15	5.03 ± 0.33	NS	NS
Liver triglyceride (mg/g tissue)	485 ± 47	552 ± 67	293 ± 14	309 ± 32	<0.01	NS
Liver free cholesterol (mg/g tissue)	1.36 ± 0.06	1.44 ± 0.08	1.46 ± 0.03	1.54 ± 0.04	NS	NS
Liver weight (g)	1.85 ± 0.12	2.09 ± 0.22	1.42 ± 0.06	1.35 ± 0.07	<0.01	NS
Liver cholesterol ester (mg/liver)	8.34 ± 1.68	11.1 ± 3.1	6.35 ± 0.35	6.76 ± 0.50	<0.01	NS
Liver triglyceride (mg/liver)	929 ± 117	1251 ± 219	424 ± 35	435 ± 665	<0.01	NS
Liver free cholesterol (mg/liver)	2.52 ± 0.21	2.99 ± 0.37	2.07 ± 0.09	2.07 ± 0.09	<0.01	NS

Data are means ± SE. The P values are generated by two-way ANOVA testing sex and genotype effect.

neutral lipid levels. The liver weights and liver triglyceride contents were similar between $Ffar1^{+/+}$ and $Ffar1^{-/-}$ mice (Table 2). Although there were sex differences, no genotypic difference was observed between $Ffar1^{+/+}$ and $Ffar1^{-/-}$ mice of either sex. The data are consistent with those in Table 1, confirming that $Ffar1^{-/-}$ mice were not protected from high-fat diet–induced hepatic steatosis.

DISCUSSION

Free fatty acids represent something of a Jekyll and Hyde in regard to metabolic disease. Although an increase in plasma fatty acid serves as one of the nutritional cues leading to increased postprandial insulin secretion, the long-term elevation of plasma free fatty acids observed in obesity is thought to contribute to reduced insulin secretion and ultimately islet dysfunction and the development of type 2 diabetes. In this context, understanding the function of FFAR1 as a cell surface receptor for fatty acids becomes very important. To this end, we generated a line of $Ffar1^{-/-}$ mice to use as a tool in studying this receptor.

The high-fat diet–induced phenotypes of our $Ffar1^{-/-}$ mice versus those reported by Steneberg et al. (5) were very different. In the Steneberg et al. (5) report, $Ffar1^{-/-}$ mice fed the high-fat diet gained similar weight and had reduced plasma glucose, insulin, and triglyceride levels compared with $Ffar1^{+/+}$ mice. The mice also had dramatically reduced hepatic steatosis as shown by histological staining of a liver slice with oil red O. In addition, the mice were reported to show improved glucose tolerance or reduced glucose-stimulated insulin secretion and reduced hepatic glucose output. Conversely, transgenic mice overexpressing FFAR1 in pancreatic islets developed diabetes. This led Steneberg et al. (5) to suggest that FFAR1 is involved in the impairment of islet function induced by chronic exposure of elevated fatty acid associated with obesity. The data would also suggest that sustained signaling through FFAR1, such as that achieved by pharmacological activation with FFAR1 agonists, might be detrimental to diabetic patients. However, we were not able to reproduce the protective phenotypes in our $Ffar1^{-/-}$ mice using two distinct high-fat diets. Under the same high-fat diet formula as those used by Steneberg et al. (5), we observed that liver triglyceride and cholesterol accumulated to a comparable degree in both $Ffar1^{+/+}$ and $Ffar1^{-/-}$ mice, and no reduction of hepatic steatosis was observed in the $Ffar1^{-/-}$ mice (Table 1). We reasoned that because the 58% Surwit diet lacks certain unsaturated fatty acids, it may not be the right diet to reflect a physiologically relevant setting. However, using a semipurified 60% high-fat diet, we still did not observe a reduction of hepatic

steatosis in the $Ffar1^{-/-}$ mice. From the oil red O staining in the Steneberg et al. study (5), it was not clear whether the reduced liver fat accumulation in the $Ffar1^{-/-}$ mice was statistically significantly lower than that in the $Ffar1^{+/+}$ mice.

The cause of the discrepancy between our study and that of Steneberg et al. (5) is not clear. One possibility may be a difference in genetic backgrounds. Our mice, although generated in a hybrid background, were backcrossed to B6 mice. The 58% high-fat diet study was carried out with mice that were backcrossed for six generations; the 60% high-fat diet study used mice backcrossed for >10 generations. The genetic background of the mice in the Steneberg et al. study (5) was reported as "backcrossed several generations." Another possibility is that FFAR1 may play a role maintaining basal metabolic homeostasis, but its effects are masked out by high-fat diet–induced metabolic stress. In our studies, we observed some detrimental phenotypes of the $Ffar1^{-/-}$ mice on a low-fat diet. For example, our $Ffar1^{-/-}$ mice had higher liver triglyceride ($P = 0.03$) on the low-fat diet (Table 1). The $Ffar1^{-/-}$ mice were also heavier, and they had slightly more food intake (Fig. 3). The results suggest that $Ffar1^{-/-}$ mice may have an unfavorable metabolic profile at basal conditions. Latour et al. (7) reported that 13-week-old male $Ffar1^{-/-}$ mice on chow diet had a significant albeit small degree of glucose intolerance. We speculate that because $Ffar1^{-/-}$ mice have a poorer profile than the $Ffar1^{+/+}$ mice to begin with, when they reach the same metabolic endpoints on a high-fat diet, the high-fat diet–induced "net change" could be viewed as smaller in the $Ffar1^{-/-}$ mice, and the smaller change could be interpreted as resistance to a high-fat diet. However, it should be emphasized that, based on the detrimental phenotypes of $Ffar1^{-/-}$ mice in the present study and the glucose intolerance data from Latour et al. (7), deletion of FFAR1 does not decrease (rather, it likely increases) the risk of metabolic disease on either low-fat diet or high-fat diet.

Another controversy in the literature is whether FFAR1 mediates the negative effects of chronic elevated fatty acids on insulin secretion and insulin resistance and whether FFAR1 links obesity and insulin resistance. Steneberg et al. (5) showed that insulin secretion from isolated $Ffar1^{-/-}$ islets was not impaired by 48-h exposure to palmitic acid and concluded that $Ffar1^{-/-}$ islets are protected from the long-term negative effect of fatty acids. They suggested that FFAR1 mediates the long-term negative effects of fatty acids and proposed that FFAR1 antagonism may represent a therapeutic strategy for obesity-associated type 2 diabetes. In contrast, Latour et al. (7)

showed that isolated islets from *Ffar1*$^{-/-}$ mice were still subject to the impairment of the insulin response after chronic exposure to fatty acids and suggested that FFAR1 mediates the acute effect of fatty acids on insulin secretion but not the chronic, deleterious effects. Additionally, *Ffar1*$^{-/-}$ mice have approximately one-half the acute insulin secretion in vivo in response to a bolus of injected Intralipid. Although the objective of our study was to examine the effects of high-fat diet on *Ffar1*$^{-/-}$ mice, we did confirm that FFAR1 mediates acute fatty acid–stimulated insulin release in vivo. FFAR1 does not play a role in adipose tissue lipolysis or leptin release, but it can affect GLP-1 level. Finally, CL-316,243 treatment stimulated glucagon release, and such an effect was attenuated in *Ffar1*$^{-/-}$ mice (Fig. 3*F'*), supporting a previous report that FFAR1 is expressed in glucagon-producing cells and that it affects glucagon secretion (11).

While the present manuscript was in revision, three new studies appeared in press in *Diabetes* addressing the roles of GPR40/FFAR1 in insulin secretion and metabolic homeostasis. The first study by Tan et al. (12) showed that selective small-molecule agonists of FFAR1 promoted glucose-dependent insulin secretion and reduced blood glucose in *Ffar1*$^{+/+}$ but not in *Ffar1*$^{-/-}$ mice, and they concluded that FFAR1 does not mediate the long-term effects of fatty acids on islet function. Our results support these findings. The second study by Edfalk et al. (13) showed that *Ffar1* is expressed in enteroendocrine cells and mediates fatty acid stimulation of incretin secretion. Our data support a possible involvement of FFAR1 in fatty acid–mediated GLP-1 secretion. Finally, the study by Kebede et al. (14) examined the role of FFAR1 in insulin secretion in vivo after high-fat feeding. They reported that *Ffar1*$^{-/-}$ mice had fasting hyperglycemia despite normal glucose and insulin tolerance. *Ffar1*$^{-/-}$ mice became as obese, became as glucose intolerant and as insulin resistant as their *Ffar1*$^{+/+}$ littermates under high-fat diet (60% fat), and developed a similar degree of liver steatosis (14). Our results are consistent with those in the Kebede et al. study (14). We also show that *Ffar1*$^{-/-}$ mice had slightly more food intake and body weight on a low-fat diet. In addition, we provide quantitative data showing that livers of *Ffar1*$^{-/-}$ mice accumulate no less neutral lipids than *Ffar1*$^{+/+}$ littermates under either low-fat diet or high-fat diet.

In conclusion, the present study demonstrates that FFAR1 contributes to the maintenance of basal metabolism and that deletion of FFAR1 does not protect mice from high-fat diet–induced metabolic disease. Our results, along with those of the recently published studies (7,12–14), jointly argue that antagonists of FFAR1 may not provide significant benefit for obesity-associated type 2 diabetes.

REFERENCES

1. Brown AJ, Jupe S, Briscoe CP: A family of fatty acid binding receptors. *DNA Cell Biol* 24:54–61, 2005
2. Itoh Y, Kawamata Y, Harada M, Kobayashi M, Fujii R, Fukusumi S, Ogi K, Hosoya M, Tanaka Y, Uejima H, Tanaka H, Maruyama M, Satoh R, Okubo S, Kizawa H, Komatsu H, Matsumura F, Noguchi Y, Shinohara T, Hinuma S, Fujisawa Y, Fujino M: Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. *Nature* 422:173–176, 2003
3. Hirasawa A, Tsumaya K, Awaji T, Katsuma S, Adachi T, Yamada M, Sugimoto Y, Miyazaki S, Tsujimoto G: Free fatty acids regulate gut incretin glucagon-like peptide-1 secretion through GPR120. *Nat Med* 11:90–94, 2005
4. Winzell MS, Ahren B: G-protein-coupled receptors and islet function: implications for treatment of type 2 diabetes. *Pharmacol Ther* 116:437–448, 2007
5. Steneberg P, Rubins N, Bartoov-Shifman R, Walker MD, Edlund H: The FFA receptor GPR40 links hyperinsulinemia, hepatic steatosis, and impaired glucose homeostasis in mouse. *Cell Metab* 1:245–258, 2005
6. Unger RH: Lipotoxic diseases. *Annu Rev Med* 53:319–336, 2002
7. Latour MG, Alquier T, Oseid E, Tremblay C, Jetton TL, Luo J, Lin DC, Poitout V: GPR40 is necessary but not sufficient for fatty acid stimulation of insulin secretion in vivo. *Diabetes* 56:1087–1094, 2007
8. Susulic VS, Frederich RC, Lawitts J, Tozzo E, Kahn BB, Harper ME, Himms-Hagen J, Flier JS, Lowell BB: Targeted disruption of the beta 3-adrenergic receptor gene. *J Biol Chem* 270:29483–29492, 1995
9. Burrier RE, Deren S, McGregor DG, Hoos LM, Smith AA, Davis HR Jr: Demonstration of a direct effect on hepatic acyl CoA: cholesterol acyl transferase (ACAT) activity by an orally administered enzyme inhibitor in the hamster. *Biochem Pharmacol* 47:1545–1551, 1994
10. Umekawa T, Yoshida T, Sakane N, Kondo M: Effect of CL316,243, a highly specific beta 3-adrenoceptor agonist, on lipolysis of human and rat adipocytes. *Horm Metab Res* 28:394–396, 1996
11. Flodgren E, Olde B, Meidute-Abaraviciene S, Winzell MS, Ahren B, Salehi A: GPR40 is expressed in glucagon producing cells and affects glucagon secretion. *Biochem Biophys Res Commun* 354:240–245, 2007
12. Tan CP, Feng Y, Zhou YP, Eiermann GJ, Petrov A, Zhou C, Lin S, Salituro G, Meinke P, Mosley R, Akiyama TE, Einstein M, Kumar S, Berger JP, Mills SG, Thornberry NA, Yang L, Howard AD: Selective small-molecule agonists of G protein-coupled receptor 40 promote glucose-dependent insulin secretion and reduce blood glucose in mice. *Diabetes* 57:2211–2219, 2008
13. Edfalk S, Steneberg P, Edlund H: Gpr40 is expressed in enteroendocrine cells and mediates FFA stimulation of incretin secretion. *Diabetes* 57:2280–2287, 2008
14. Kebede M, Alquier T, Latour MG, Semache M, Tremblay C, Poitout V: The fatty-acid receptor GPR40 plays a role in insulin secretion in vivo after high-fat feeding. *Diabetes* 57:2432–2437, 2008