Molecular Therapy for Obesity and Diabetes Based on a Long-Term Increase in Hepatic Fatty-Acid Oxidation

Josep M. Orellana-Gavaldà,[1] Laura Herrero,[1] Maria Ida Malandrino,[1] Astrid Pañeda,[2] Maria Sol Rodríguez-Peña,[2] Harald Petry,[2] Guillermina Asins,[1] Sander Van Deventer,[2] Fausto G. Hegardt,[1] and Dolors Serra[1]

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Obesity-induced insulin resistance is associated with both ectopic lipid deposition and chronic, low-grade adipose tissue inflammation. Despite their excess fat, obese individuals show lower fatty-acid oxidation (FAO) rates. This has raised the question of whether burning off the excess fat could improve the obese metabolic phenotype. Here we used human-safe nonimmunoreactive adeno-associated viruses (AAV) to mediate long-term hepatic gene transfer of carnitine palmitoyltransferase 1A (CPT1A), the key enzyme in fatty-acid *β*-oxidation, or its permanently active mutant form CPT1AM, to high-fat diet-treated and genetically obese mice. High-fat diet CPT1A- and, to a greater extent, CPT1AM-expressing mice showed an enhanced hepatic FAO which resulted in increased production of CO_2, adenosine triphosphate, and ketone bodies. Notably, the increase in hepatic FAO not only reduced liver triacylglyceride content, inflammation, and reactive oxygen species levels but also systemically affected a decrease in epididymal adipose tissue weight and inflammation and improved insulin signaling in liver, adipose tissue, and muscle. Obesity-induced weight gain, increase in fasting blood glucose and insulin levels, and augmented expression of gluconeogenic genes were restored to normal only 3 months after AAV treatment. Thus, CPT1A- and, to a greater extent, CPT1AM-expressing mice were protected against obesity-induced weight gain, hepatic steatosis, diabetes, and obesity-induced insulin resistance. In addition, genetically obese db/db mice that expressed CPT1AM showed reduced glucose and insulin levels and liver steatosis. *Conclusion*: A chronic increase in liver FAO improves the obese metabolic phenotype, which indicates that AAV-mediated CPT1A expression could be a potential molecular therapy for obesity and diabetes. (HEPATOLOGY **2010;000:000-000.**)

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Obesity is a major risk factor for disorders ranging from insulin resistance and type 2 diabetes (T2D) to hepatic steatosis and cardiovascular disease. The incidence of obesity is increasing worldwide and a concerted effort is being made to understand its pathogenesis. Two main mechanisms have been proposed to explain obesity-induced insulin resistance: on the one hand the ectopic deposition of triacylglyceride

Abbreviations: AAV, adeno-associated viruses; ACC1, acetyl-CoA carboxylase 1; ASP, acid-soluble products; BHB-CoA, β-hydroxybutyryl-CoA; CPT1A, carnitine palmitoyltransferase I liver isoform; DGAT2, diacylglycerol O-acyltransferase homolog 2; GFP, green fluorescent protein; G6Pase, glucose-6-phosphatase; HFD, high-fat diet; HMGS2, hydroxymethylglutaryl-CoA synthase 2; MCD, malonyl-CoA decarboxylase; MCP-1, monocyte chemoattractant protein-1; MTP, microsomal triacylglycerol transfer protein; PDK4, pyruvate dehydrogenase kinase-4; SCD1, stearoyl-Coenzyme A desaturase 1; TAG, triacylglyceride; UCP1, uncoupling protein 1; VLCAD, very long-chain acyl-CoA dehydrogenase.

From the [1]Department of Biochemistry and Molecular Biology, Institut de Biomedicina de la Universitat de Barcelona (IBUB) and CIBER Fisiopatologia de la Obesidad y Nutrición (CIBEROBN), 08028 Barcelona, Spain; and [2]Amsterdam Molecular Therapeutics, Amsterdam, The Netherlands.

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Current address for Astrid Pañeda: Division of Gene Therapy and Hepatology, Center for Applied Medical Research, University of Navarra, Pamplona, Spain.

AQ1 *Address reprint requests to: Dolors Serra, Ph.D., Department of Biochemistry and Molecular Biology, School of Pharmacy, University of Barcelona, Av. Diagonal 643, E-08028 Barcelona, Spain. E-mail: dserra@ub.edu; fax: (34) 934 024 520.*

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(TAG) outside the adipose tissue,[1] and on the other, the heightened inflammatory state of the adipose tissue and liver.[2]

However, the ultimate cause of obesity is an energy imbalance between intake and expenditure, leading to the accumulation of excess nutrients in lipid deposits. Therefore, any strategy able to tilt the balance towards fatty-acid oxidation (FAO) could improve obesity-induced disorders. Malonyl-CoA, derived from glucose metabolism and the first intermediate in lipogenesis, regulates FAO by inhibiting carnitine palmitoyltransferase 1 (CPT1). This makes CPT1 the rate-limiting step in mitochondrial fatty-acid β-oxidation. Short-term genetic studies that increased FAO in liver showed a decrease in hepatic TAG content[3] and insulin resistance in obese rodents.[4,5] However, to date there is no successful approach to chronically increase FAO and improve whole-animal obesity-induced insulin resistance *in vivo*.

Here we achieved hepatic gene transfer of CPT1A (CPT1 liver isoform) to obese mice by injecting adeno-associated viruses (AAV) into the tail vein. This led to a nonimmunoreactive, long-term increase in lipid oxidation. We also used a mutant but active form of CPT1A (CPT1AM[6]), which is insensitive to malonyl-CoA and therefore leads to a permanent increase in the rate of FAO, independently of the glucose-derived malonyl-CoA levels. Our results show that an increase in hepatic FAO through AAV-mediated gene transfer of CPT1A and CPT1AM reduced obesity-induced hepatic steatosis, weight gain, inflammation, diabetes, and insulin resistance in mice consuming a high-fat diet (HFD). Furthermore, CPT1AM expression also reduced glucose and insulin levels, and liver steatosis in genetically obese *db/db* mice.

Materials and Methods

Adeno-Associated Vectors. AAV vectors, serotype 1, AAV1-AAT-GFP, AAV1-AAT-CPT1A, and AAV1-AAT-CPT1AM were constructed to drive mouse liver expression of green fluorescent protein (GFP), CPT1A, and CPT1AM, respectively. Vector plasmids carried the human albumin enhancer element and the human 1-antitrypsin (EalbAATp) liver-specific promoter described by Kramer et al.[30]; the cDNA sequence of GFP, CPT1A,[31] and CPT1AM[6]; the woodchuck posttranscriptional regulatory element (WPRE, Access. No. AY468-486)[32]; and the bovine growth hormone polyadenosine transcription termination signal [bGH-poly(A)] (bases 2326-2533 GenBank Access. No. M57764). The expression cassette was flanked by two inverted terminal repeats (ITRs) derived from AAV2. AAV1 vectors were produced in insect cells using baculovirus.[33] The vector preparations used had titers of 1×10^{12}, 7.6×10^{11}, and 7.5×10^{11} genome copies (gc)/ml for AAV1-AAT-GFP, AAV1-AAT-CPT1A, and AAV1-AAT-CPT1AM respectively.

Animals. Eight-week-old male C75Bl/6J mice were fed for 10-15 weeks with either NCD (TestDiet D8Y2, 10% Kcal fat) or HFD (TestDiet D8Y1, 60% Kcal fat). Two weeks after diet treatment, AAV1 vectors were administered by tail vein injection in a single dose of 7.5×10^{12} gc/kg of body weight. Mice were killed 4 to 13 weeks after virus injection. Eight-week-old male C75BL/KsJ-db/db and C75BL/KsJ-db/+ control mice were injected with AAV1 vectors in the tail vein at a single dose of 7.5×10^{12} gc/kg and killed 17 weeks later.

Methods. Primary mouse hepatocytes were isolated by the collagenase method[33] and used to measure FAO to CO_2.[34] Isolation of mitochondria from liver was obtained as described.[35] Measurement of CPT1 activity was determined by the radiometric method.[36] Glucose and pyruvate tolerance tests (2.0 g per kg body weight) were administered by intraperitoneal injection after an overnight fast. Histological examination was done using formalin-fixed, paraffin-embedded tissue sections stained with hematoxylin-eosin at the Pathology Department of the Hospital Clinic of Barcelona.

Statistical Analysis and Other Methods. Data are presented as mean ± SEM. Student's *t* test was used for statistical analysis. Differences were considered significant at $P < 0.05$ and complete methods are described in the Supporting Information.

Results

AAV1-Mediated Expression of CPT1A and CPT1AM in Mouse Liver Increases FAO. To deliver CPT1A, CPT1AM, and GFP as a control to the mouse liver we prepared three adeno-associated viruses: AAV1-CPT1A, AAV1-CPT1AM, and AAV1-GFP. The genome of each virus contained the target gene under the control of a liver-specific promoter EalbAATp (Fig. 1A). AAVs were administrated by the tail-vein injection to C57Bl/6J mice that had been feeding for 2 weeks on either normal chow diet (NCD) or HFD. Mice were studied throughout the dietary treatment and killed at either 4 or 13 weeks after AVV administration, for short- or long-term studies, respectively (Fig. 1A). Long-term expression of the virus was

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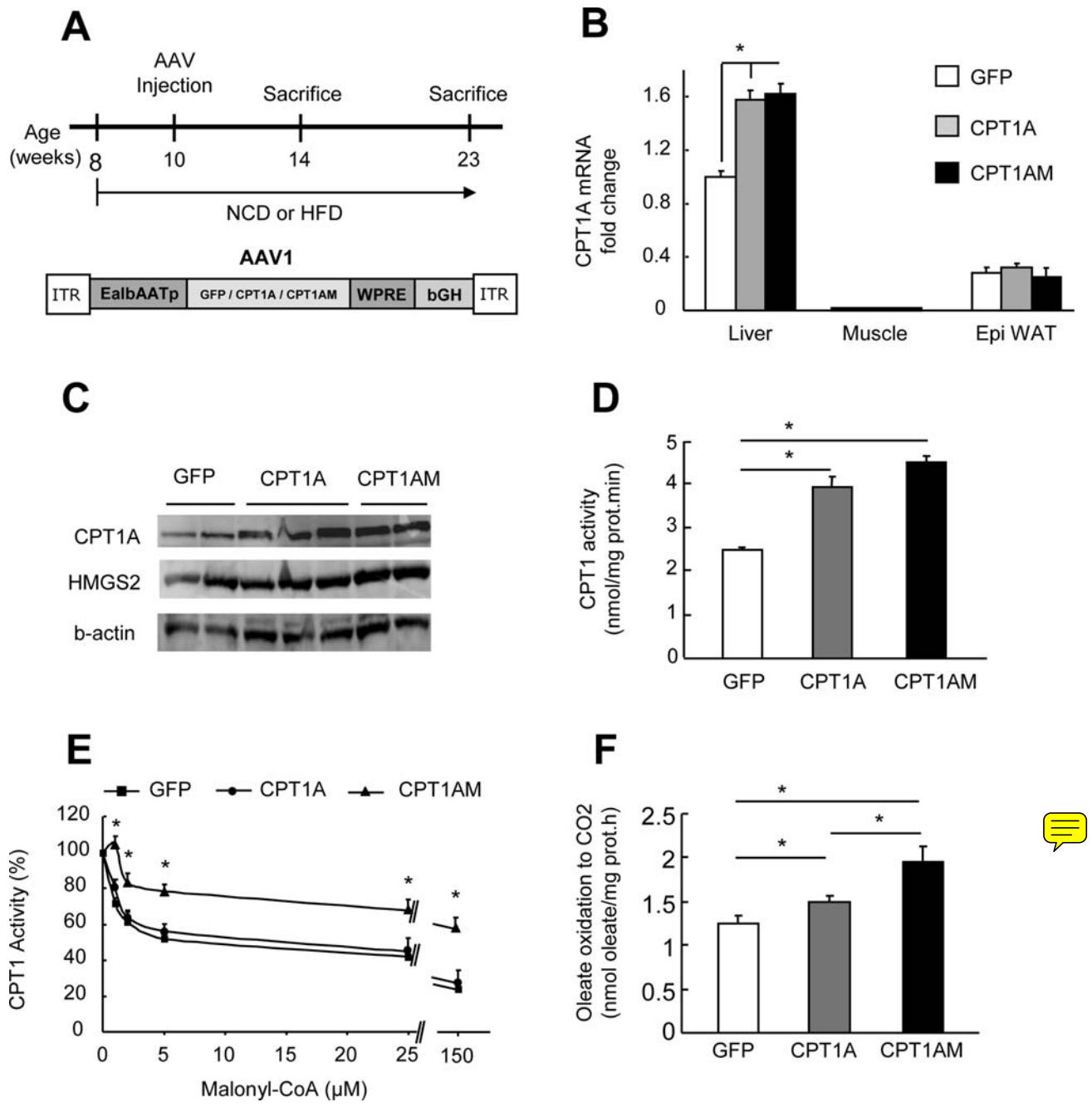

Fig. 1. Increased CPT1A mRNA, protein, activity, and oleate oxidation in CPT1A- and CPT1AM-expressing mice on HFD. (A) Experiment time course and schema of the AAV vectors: AAV-GFP; AAV-CPT1A, and AAV-CPT1AM. The cassettes contain the GFP, CPT1A, or CPT1AM transgene driven by liver specific EalbAATp promoter. (B) CPT1A mRNA expression in liver, epididymal white adipose tissue (epi WAT), and muscle from 14-week-old HFD-treated mice. Primers recognize both CPT1A and CPT1AM sequences. (C) Protein levels from liver mitochondria of HFD mice. (D,E) CPT1 activity from isolated liver mitochondria of HFD-fed mice and CPT1 activity from NCD liver mitochondria enriched cell fractions incubated with different amounts of malonyl-CoA. Data are means ± SEM of six experiments. (F) Oleate oxidation to CO_2 from primary hepatocytes isolated from HFD GFP-, CPT1A-, or CPT1AM-expressing mice. Experiments were done at 4 weeks after the virus treatment. Shown are representative experiments performed in triplicate. n = 6-10. *$P < 0.05$. **$P < 0.05$ versus GFP.

evaluated in mice injected with AAV-GFP until they were 33 weeks old (Supporting Fig. 1A-D). Specific expression of CPT1A and CPT1AM in the liver was measured by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) (Fig. 1B). CPT1A mRNA expression levels were 58% and 62% higher in liver of CPT1A- and CPT1AM-expressing mice, respectively, compared to GFP control mice. No significant differences were seen in other tissues such as muscle or white adipose tissue (Fig. 1B). Liver CPT1 protein and activity levels were increased in those animals injected with AAV-CPT1A and AAV-CPT1AM

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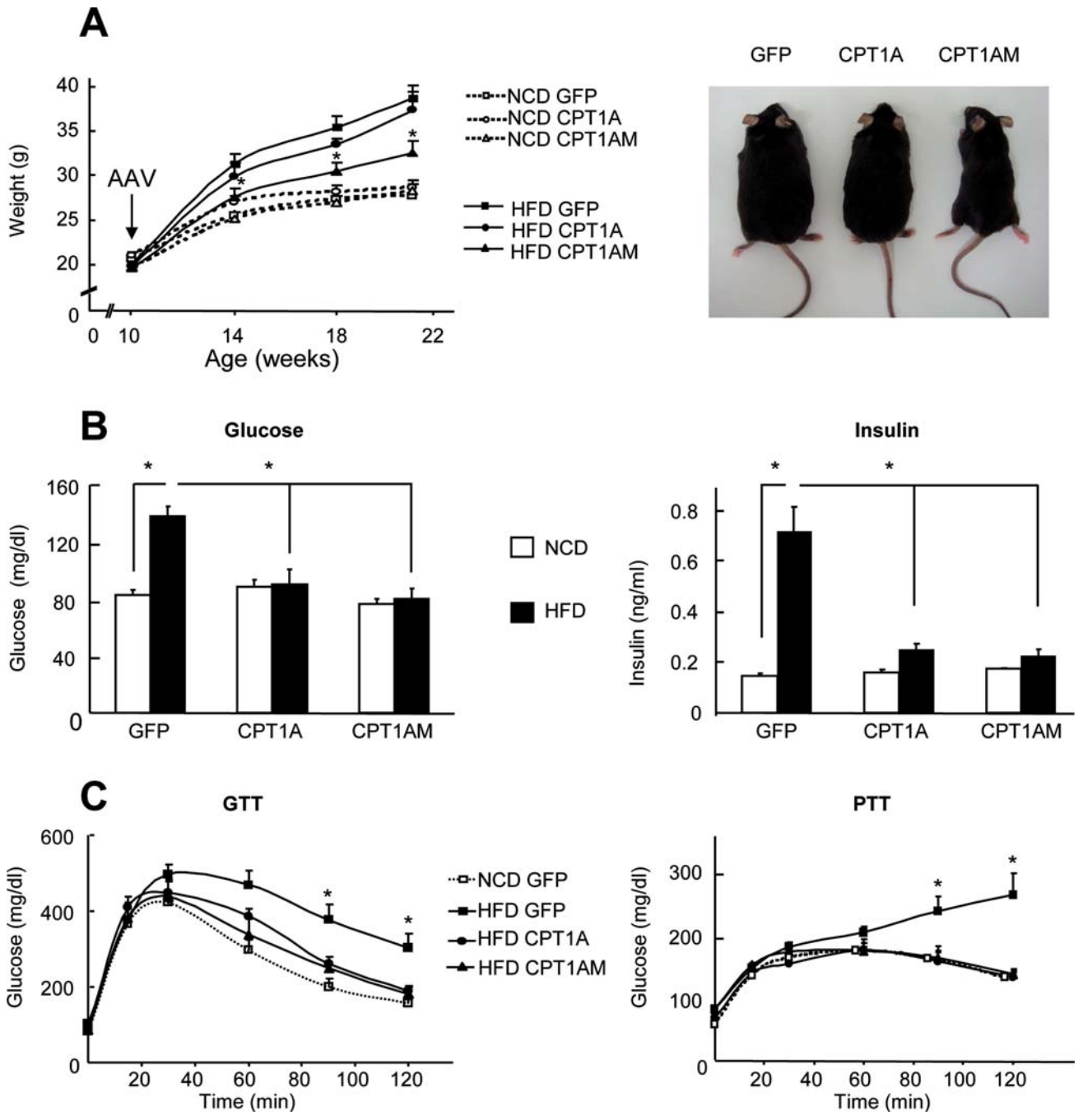

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Fig. 2. Metabolic parameters in CPT1A- and CPT1AM-expressing mice. (A) Body weight. *$P < 0.05$ HFD AAV-CPT1AM versus NCD AAV-GFP, left panel and mice receiving CPT1A, and CPT1AM remained leaner than control 3 months after AAV injection and HFD treatment, right panel. (B) Fasting blood glucose, left panel and insulin levels, right panel. (C) Glucose tolerance test, left panel and pyruvate tolerance test, right panel. n = 6-10. *$P < 0.05$.

compared to control AAV-GFP in both HFD (Fig. 1C,D) and NCD (Supporting Fig. 2A,B). Liver protein levels increased 3.08 ± 0.2- and 3.01 ± 0.15-fold in HFD CPT1A-, and CPT1AM-expressing mice, respectively, compared to GFP control mice (Supporting Fig. 1E). CPT1 activity was also higher in HFD CPT1A-, and CPT1AM-expressing mice compared to GFP control mice (GFP: 2.51 ± 0.07, CPT1A: 3.96 ± 0.25, and CPT1AM: 4.53 ± 0.15 nmol.mg prot^{-1}.min^{-1}; $P < 0.05$) (Fig. 1D).

CPT1AM is not inhibited by malonyl-CoA in yeast, pancreatic β-cells, muscle cells, or primary rat hepatocytes.[6-9] We measured CPT1 activity in the presence of increasing concentrations of malonyl-CoA in liver

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Table 1. Daily Food Intake and Metabolic Variables in Serum and Liver

	NCD			HFD		
	AAV-GFP	AAV-CPT1A	AAV-CPT1AM	AAV-GFP	AAV-CPT1A	AAV-CPT1AM
Daily food intake (g)	3.43 ± 0.14	3.68 ± 0.12	3.49 ± 0.14	2.72 ± 0.15*	2.91 ± 0.19	2.52 ± 0.13
Serum						
FFA (mM)	0.94 ± 0.1	1.23 ± 0.08	1.24 ± 0.16	1.34 ± 0.08*	1.05 ± 0.07†	1.11 ± 0.08†
TAG (mg/dl)	82.97 ± 6.90	84.31 ± 3.20	78.61 ± 2.70	113.79 ± 10.00*	114.09 ± 9.3	89.96 ± 8.3† ‡
BHB (mM)	1.40 ± 0.04	1.74 ± 0.04†	1.82 ± 0.17†	1.57 ± 0.1*	1.59 ± 0.06	1.90 ± 0.13† ‡
Liver						
BHB-CoA (nmol/g liver)	7.4 ± 0.8	9.7 ± 1.1	12.6 ± 1.3†	6.8 ± 0.9	10.2 ± 1.8	14.6 ± 1.7†
ATP (nmol/g liver)	6.9 ± 0.5	15.4 ± 1.6†	25.1 ± 2.4† ‡	7.2 ± 0.8	16.8 ± 1.2†	23.2 ± 2.8† ‡

Daily food intake and circulating free fatty acids (FFA) and triacylglycerides (TAG) and β-hydroxybutyrate (BHB), and β-hydroxybutyrate-CoA (BHB-CoA) and ATP from 16-week-old overnight fasted mice. Average food intake was calculated for the period between 8 to 14 weeks of age. Data are represented as mean ± S.E.M.

*$P < 0.05$ AAV-GFP HFD versus NCD.

†$P < 0.05$ compared to AAV-GFP on the same diet.

‡$P < 0.05$ compared to AAV-CPT1A versus AAV-CPT1AM on the same diet.

mitochondrion-enriched fractions of GFP-, CPT1A-, and CPT1AM-expressing mice. At physiological concentrations of malonyl-CoA (1 to 10 μM), CPT1AM-expressing mice retained up to 78% of their activity, whereas GFP- and CPT1A-expressing mice retained only 48% (Fig. 1E). This indicates that cells expressing CPT1AM will retain most of their CPT1 activity independently of the malonyl-CoA levels. Notably, liver malonyl-CoA levels were similar for GFP-, CPT1A-, and CPT1AM-expressing mice fed on either NCD or HFD (Supporting Fig. 1F).

Next, we examined whether the increase in CPT1 messenger RNA (mRNA), protein, and activity seen in CPT1A- and CPT1AM-expressing mice affected fatty-acid β-oxidation. We isolated primary hepatocytes from GFP-, CPT1A-, and CPT1AM-expressing mice treated with NCD or HFD and measured [1-^{14}C]oleate oxidation to CO_2 and acid-soluble products (ASPs), mainly ketone bodies. In HFD-treated mice, FAO to CO_2 increased by 20.9% ± 0.8%, and 56.4% ± 4.6% in CPT1A- and CPT1AM-expressing mice, respectively, compared to GFP control mice (Fig. 1F). Similar results were obtained for FAO to ASP and total FAO (the sum of oxidation to CO_2 and ASP) in HFD-treated mice (Supporting Fig. 2C,D) and in NCD-treated mice (Supporting Fig. 2E-G). Importantly, oxidation rates were higher in CPT1AM- than in CPT1A-expressing mice, consistent with the higher efficiency of CPT1AM independently of the glucose-derived malonyl-CoA concentrations.

Long-chain fatty-acids undergoing β-oxidation yield acetyl-CoA moieties that have two main possible fates: (1) entry to the Krebs cycle for complete oxidation and adenosine triphosphate (ATP) production, or (2) conversion to ketone bodies. We hypothesized that accelerated β-oxidation due to CPT1A expression could reduce the surplus of acetyl-CoA groups by way of both pathways. Liver ATP levels of CPT1A- and CPT1AM-expressing mice were increased compared to control GFP mice both in NCD and HFD (Table 1). T1
Liver protein levels of mitochondrial hydroxymethyl-glutaryl-CoA synthase 2 (HMGS2), the rate-limiting enzyme of hepatic ketogenesis, were increased in CPT1A-, and CPT1AM-expressing mice compared to control (Fig. 1C; Supporting Figs. 1E, 2A). Consistent with this, liver and serum levels of ketone bodies such as β-hydroxybutyryl-CoA (BHB-CoA) were higher in CPT1A- and CPT1AM-expressing mice than in GFP control mice both in NCD or HFD (Table 1).

CPT1A and CPT1AM Expression Protected from Obesity-Induced Weight Gain and Insulin Resistance. We next examined the effects of increased β-oxidation on the obese metabolic phenotype. Mice injected with AAV-GFP, AAV-CPT1A, or AAV-CPT1AM were studied under HFD treatment. Although no weight differences were seen in CPT1A- or CPT1AM-expressing mice on NCD, CPT1AM-expressing mice on HFD weighed significantly less than control mice 11 weeks after AAV infection (GFP: 38.7 ± 1.4 g, CPT1AM: 32.5 ± 1.3 g; $P < 0.04$) (Fig. 2A). Interestingly, CPT1AM-expressing mice showed a stronger anti-obesity effect than CPT1A-expressing mice, most likely due to the higher FAO rate observed in the former. The differences in weight gain were not attributable to differences in food consumption because daily rates of food intake were equal in GFP-, CPT1A-, and CPT1AM-expressing mice (Table 1). Notably, fasting blood glucose concentrations (GFP: 128.6 ± 18.0, CPT1A: 87.2 ± 10.7, and CPT1AM: 82.0 ± 7.1 mg/dL; $P < 0.05$) and insulin

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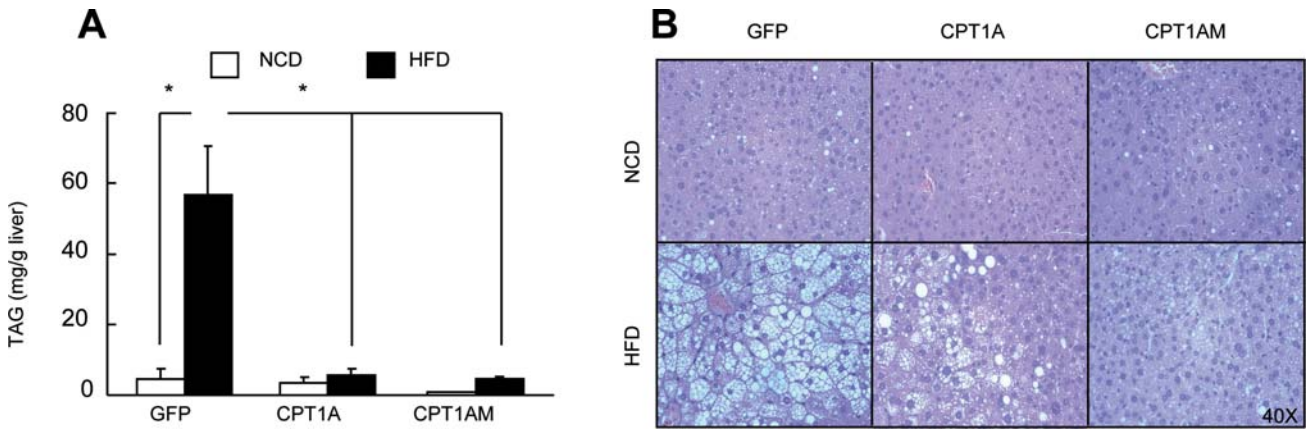

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Fig. 3. Liver TAG content and liver histology in CPT1A- and CPT1AM-expressing mice. (A) Liver TAG content of 14-week-old GFP, CPT1A-, and CPT1AM-expressing mice. (B) Liver histological sections (hematoxylin and eosin [H&E] staining) from representative 29-week-old GFP-, CPT1A-, and CPT1AM-expressing littermates. n = 6-10. *$P < 0.05$.

levels (GFP: 0.72 ± 0.10, CPT1A: 0.25 ± 0.02, and CPT1AM: 0.22 ± 0.02 ng/mL; $P < 0.04$) were lower in both CPT1A-, and CPT1AM-expressing mice than in control mice on HFD, and similar to the levels found in the mice on NCD (Fig. 2B). Glucose tolerance (measured by way of an intraperitoneal GTT; Fig. 2C, left panel) and gluconeogenesis (measured by way of an intraperitoneal injection of pyruvate; Fig. 2C, right panel) were lower in both CPT1A- and CPT1AM-expressing mice than in HFD control mice. Thus, CPT1A and CPT1AM expression improved the obesity-induced diabetic and insulin-resistant phenotype.

We then examined the effect of the higher FAO levels in CPT1A- and CPT1AM-expressing mice on liver steatosis. Liver TAG content of HFD CPT1A- and CPT1AM-expressing mice was lower than that of HFD control mice (Fig. 3A). Consistent with this, circulating levels of free fatty acids (FFA) and TAG were also reduced (Table 1). Although liver from HFD control mice showed severe centrilobular steatosis, those of CPT1A-, and to a greater extent CPT1AM-expressing mice, were clearly improved (Fig. 3B). CPT1A- and CPT1AM-expression did not affect liver histology in NCD mice (Fig. 3B).

AAV-CPT1A and AAV-CPT1AM-Treatment Reverted the HFD-Induced Increase in Hepatic Gluconeogenesis, Inflammation, and Reactive Oxygen Species (ROS) Levels. We next examined the mechanisms by which accelerated FAO in CPT1A- and CPT1AM-expressing mice improved obesity-induced diabetes and insulin resistance. Four weeks after virus injection hepatic mRNAs levels of genes involved in gluconeogenic, lipogenic, and inflammatory pathways were analyzed. At this short time, glucose (data not shown) and body weight (Fig. 2A) values were already normalized in HFD CPT1AM-expressing mice compared to HFD control mice. mRNA levels of glucose-6-phosphatase (G6Pase) and pyruvate dehydrogenase kinase-4 (PDK4), which are involved in the gluconeogenic and glycolytic pathways, were increased under HFD treatment (Fig. 4A). The increase in G6Pase and PDK4 expression attributed to HFD was restored to NCD values in CPT1A- and CPT1AM-expressing mice. No changes were observed in PEPCK mRNA levels (Supporting Fig. 3A). We next looked at lipogenic enzymes such as acetyl-CoA carboxylase 1 (ACC1), diacylglycerol O-acyltransferase homolog 2 (DGAT2), and the VLDL secretory enzyme microsomal triacylglycerol transfer protein (MTP). ACC1 and DGAT2 expression was lower in the HFD group, but this decrease was restored in CPT1A- and CPT1AM-expressing mice (Fig. 4C). Similar results were seen for other lipogenic genes such as stearoyl-Coenzyme A desaturase 1 (SCD1) and AAC2 mRNA levels (Supporting Fig. 3B,C). Correlating with *de novo* lipogenesis normalization, the HFD-increase of MTP mRNA levels seen in GFP control mice was blunted in CPT1A- and CPT1AM-expressing mice, in which values returned to NCD control levels (Fig. 4C). These results indicated that the increase in liver FAO observed in CPT1A- and CPT1AM-expressing mice improved liver glucose and lipid metabolism.

Obesity-induced insulin resistance has been associated with chronic, low-grade inflammation in liver and adipose tissue.[2] To investigate the involvement of inflammation in the improvement of insulin resistance in CPT1A- and CPT1AM-expressing mice, we measured mRNA levels of several proinflammatory markers. mRNA levels for tumor necrosis factor alpha (TNFα), interleukin (IL)-6, and IL-1α increased 1.56-, 2.30-,

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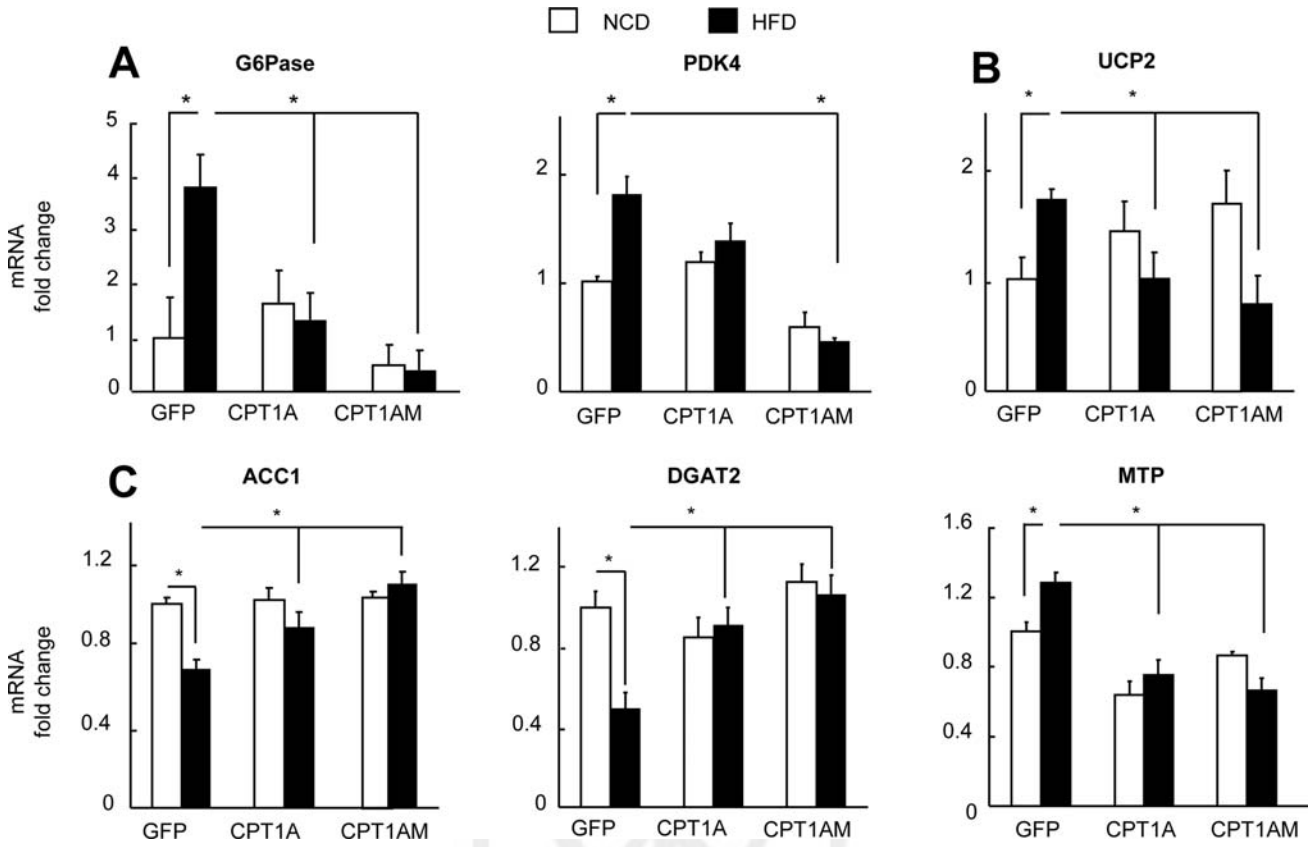

Fig. 4. Liver gene expression. (A-C) mRNA expression in liver of 14-week-old GFP-, CPT1A-, and CPT1AM-expressing mice on NCD (open bars) versus HFD (filled bars). n = 6-10. *$P < 0.05$.

and 4.86-fold, respectively, in HFD GFP control mice versus NCD ($P < 0.04$) (Fig. 5A). Importantly, these values were restored to NCD values in both CPT1A- and CPT1AM-expressing mice. Similar results were obtained for iNOS, SOCS3, and MCP-1 (Supporting Fig. 3D). Thus, CPT1A and CPT1AM expression and the concomitant increase in FAO reduced obesity-induced inflammatory stress in the liver. Oxidative stress can cause inflammation.[10] Thus, we next evaluated the mRNA expression of the uncoupling protein UCP2, a thermogenic protein and a marker of oxidative stress,[11] and ROS liver levels. Although HFD increased liver UCP2 mRNA levels in control mice, this increase was blunted in CPT1A- and CPT1AM-expressing mice (Fig. 4B). These results are consistent with liver ROS levels analyzed from GFP-, CPT1A-, and CPT1AM-expressing mice under NCD or HFD treatment. HFD increased ROS levels by 77.29% ± 12.33 ($P < 0.05$) in control mice (Fig. 5B). However, ROS levels in CPT1A- and CPT1AM-expressing mice were not significantly different from NCD values.

Altogether, our results indicate that the mechanisms by which CPT1A- and CPT1AM-expressing mice improved obesity-induced insulin resistance and diabetes involve a decrease in gluconeogenesis, restoration of fatty-acid synthesis levels, and decreased inflammatory and ROS levels.

Systemic Effect of Liver CPT1A and CPT1AM Expression. We examined the systemic effect of a chronic increase in liver FAO in adipose tissue. Epididymal adipose tissue weight from CPT1A- and CPT1AM-expressing mice on HFD was reduced by 34.57% ± 7.9%, and 68.15% ± 3.9%, respectively, compared to HFD GFP control mice ($P < 0.01$) (Fig. 6A). The stronger decrease in the epididymal fat pad from CPT1AM-expressing mice is consistent with their higher rate of liver FAO (Fig. 1F). Concordant with the decrease in the adipose tissue weight, leptin serum levels from HFD CPT1A- and CPT1AM-expressing mice were reduced 1.8- and 2.6-fold, respectively, compared to HFD GFP control mice ($P < 0.04$) (Fig. 6B).

Obese adipose tissue is characterized by enlarged adipocytes together with an increase in mononuclear cell infiltration.[12-14] Mononuclear cell infiltration was lower in HFD CPT1A-expressing mice, and almost undetectable in HFD CPT1AM-expressing mice (Fig. 6C). Consistent with this, expression of

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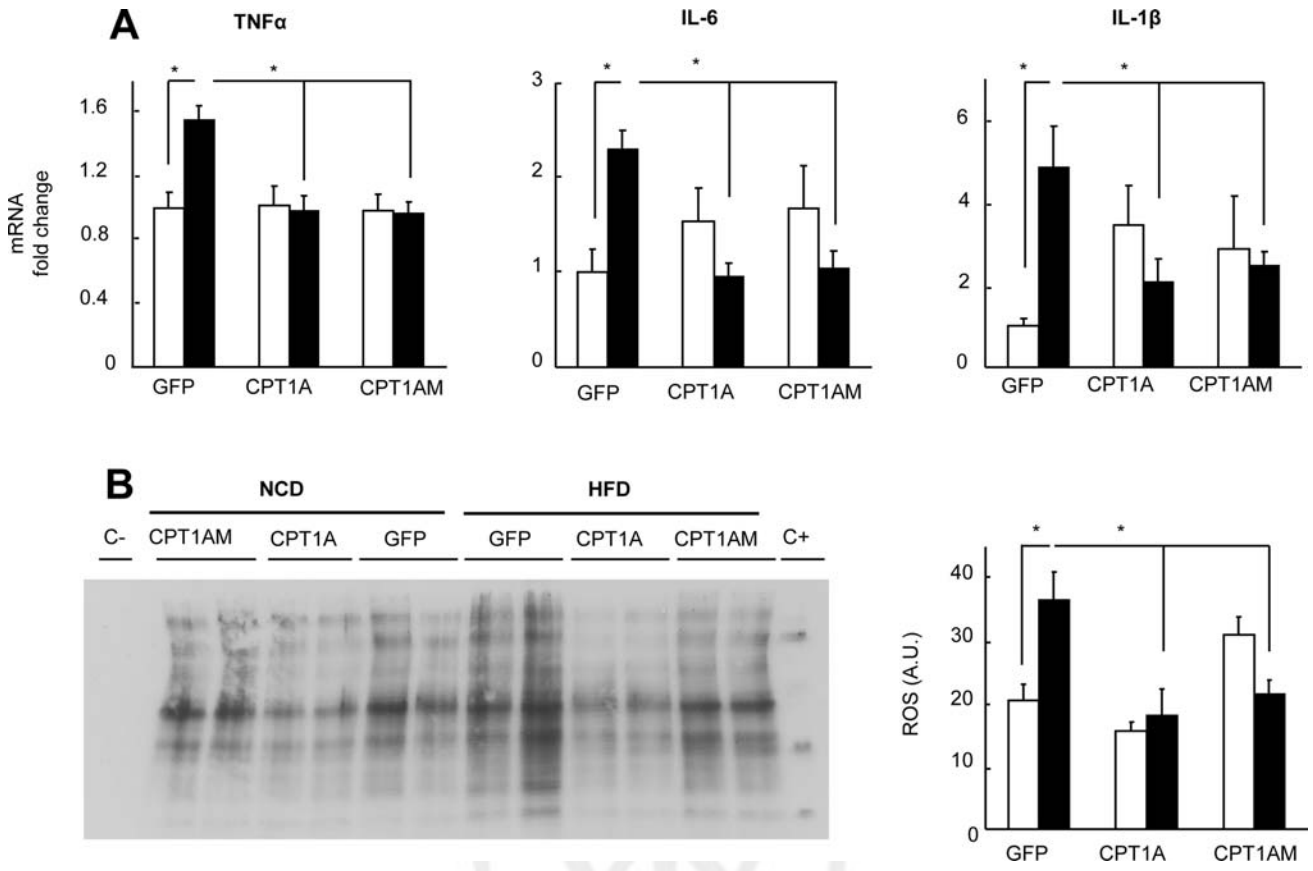

Fig. 5. Liver gene expression and ROS levels. (A) mRNA expression in liver of 14-week-old GFP-, CPT1A-, and CPT1AM-expressing mice on NCD (open bars) versus HFD (filled bars). (B) Analysis and quantification of ROS from liver total extract of 14-week-old GFP-, CPT1A-, and CPT1AM-expressing mice on NCD (open bars) and HFD (filled bars). A.U., arbitrary units. n = 6-10. *$P < 0.05$.

proinflammatory markers such as TNFα, IL-6, and MCP-1 was lower in epididymal fat pads from HFD CPT1A- and CPT1AM-expressing mice than in HFD GFP control mice (Fig. 6D-F).

The effect of an increase in hepatic FAO on insulin signaling was evaluated in liver, adipose tissue, muscle, and spleen. Interestingly, HFD-induced reduction of insulin-stimulated AKT phosphorylation was improved in CPT1A- and CPT1AM-expressing mice not only in liver but also in epididymal adipose tissue and muscle (Fig. 7A). No differences were seen in spleen. This is consistent with the improvements in glucose and insulin levels seen in these mice (Fig. 2B,C).

AAV-CPT1AM-Treatment Improved Obesity-Induced Insulin Resistance and Diabetes in db/db Mice. Because CPT1AM expression gave the strongest effect in terms of FAO, we examined the effect of AAV-CPT1AM-treatment on genetically obese mice. AAV-GFP or AAV-CPT1AM was injected into 8-week-old db/db and db/+ control mice and the metabolic phenotype was analyzed 3 months later. CPT1AM treatment reduced glucose by 41.2% ± 3.5% and insulin levels by 51.3% ± 4.6% in db/db mice (Fig. 7B,C). Hepatic steatosis was reduced (Fig. 7D) but no differences were seen in epididymal adipose tissue (Supporting Fig. 3F).

Discussion

It is widely accepted that pharmacological or genetic strategies to enhance FAO may be beneficial for the treatment of obesity and T2D. Shulman and colleagues[1,17] showed that increasing FAO can ameliorate insulin resistance by reducing hepatic and intramyocellular lipid levels. However, increased rates of FAO in muscle have also been associated with skeletal muscle insulin resistance[18,19] due to mitochondrial overload and incomplete FAO.[20] Furthermore, recent studies from Hoehn et al.[21] reported that an increase of FAO has little effect on adiposity and weight gain in mice fed HFD. These findings raise questions about whether strategies that increase FAO *per se* are sufficient to reduce whole-body adiposity *in vivo,* and which are the most appropriate tissue and gene targets. The data presented here support the notion that increased flux of fatty acids exclusively into liver mitochondria by chronic overexpression of the

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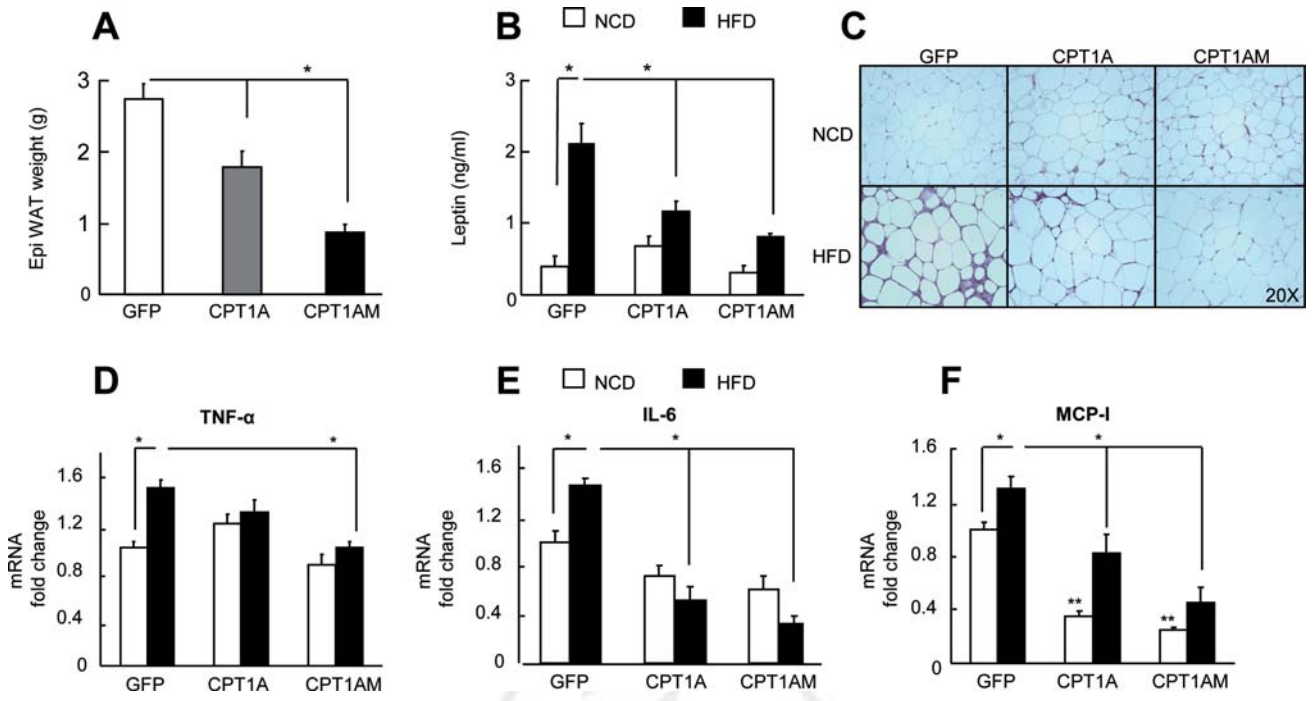

Fig. 6. Adipose tissue weight, circulating leptin, histology, and gene expression. (A) Epididymal white adipose tissue (Epi WAT) weight from GFP-, CPT1A-, and CPT1AM-expressing mice after 11 weeks on HFD. (B) Circulating leptin concentrations from GFP-, CPT1A-, and CPT1AM-expressing mice on NCD (open bars) or HFD (filled bars). (C) Histological sections from epididymal adipose tissue from a representative GFP-, CPT1A-, and CPT1AM-expressing littermates stained with H&E. (D-F) mRNA expression in adipose tissue of 14-week-old GFP-, CPT1A-, and CPT1AM-expressing mice on NCD (open bars) and HFD (filled bars). n = 6-10. *$P < 0.05$. **$P < 0.05$ NCD AAV-GFP versus NCD AAVCPT1A or AAV-CPT1AM.

COLOR

β-oxidation key enzyme, CPT1A, protects against obesity-induced insulin resistance and T2D.

The beneficial effects of CPT1A and CPT1AM gene transfer reported here were mainly the consequence of three key factors. First, the use of AAV for long-term gene expression. Recombinant AAVs are attractive candidates for use as human gene therapy vehicles because they may overcome the problem of preexisting immunity[22,23] against human AAV serotypes and produce long-term expression of the target genes. Second, the choice of the liver as a target organ, because it plays a central role in both energy expenditure and lipid/glucose homeostasis. And third, the use of a mutant but active form of CPT1A (CPT1AM[6]), which is insensitive to its physiological inhibitor, malonyl-CoA. We and others have shown that expression of CPT1AM leads to a permanent rise in the rate of FAO, independently of the glucose-derived malonyl-CoA levels.[7-9] Overall, the use of AAV-CPT1A and AAV-CPT1AM led to a long-term liver-selective gene transfer that allowed us to evaluate the metabolic impact and underlying mechanisms of increased FAO in HFD and genetically obese mice.

HFD CPT1A- and CPT1AM-expressing mice showed general improvement in hepatic glucose and lipid metabolism as a consequence of increased hepatic fatty acid flux through mitochondria. This, in turn, prevented intracellular lipid accumulation in liver and adipose tissue, especially in CPT1AM-expressing mice. However, increased fatty acid flux in the absence of a concomitant dissipation of FAO metabolites has been associated with enhanced ROS production[24] and a consequent inflammatory state.[10,25,26] Interestingly, CPT1A- and CPT1AM-expressing mice on HFD had normalized liver ROS levels and inflammatory state in both liver and adipose tissue, with a significant decrease in proinflammatory mediators such as TNFα, IL-6, and MCP-1. These results suggest that factors other than a chronic FAO increase *per se* are responsible for ROS production and inflammation. Accumulation of toxic substances (DAG or ceramides),[27] hypoxia,[28] as well as adipose tissue-derived cytokines[2] might participate in the induction of ROS production and inflammation. On the other hand, the beneficial effect of an increased FAO rate observed in HFD CPT1A- and CPT1AM-expressing mice might also be attributed to a concomitant enhancement of hepatic ketone body production (present data and[29]). Although to a lesser extent, increased FAO to CO_2, ATP, and ASPs was also observed in NCD CPT1A-

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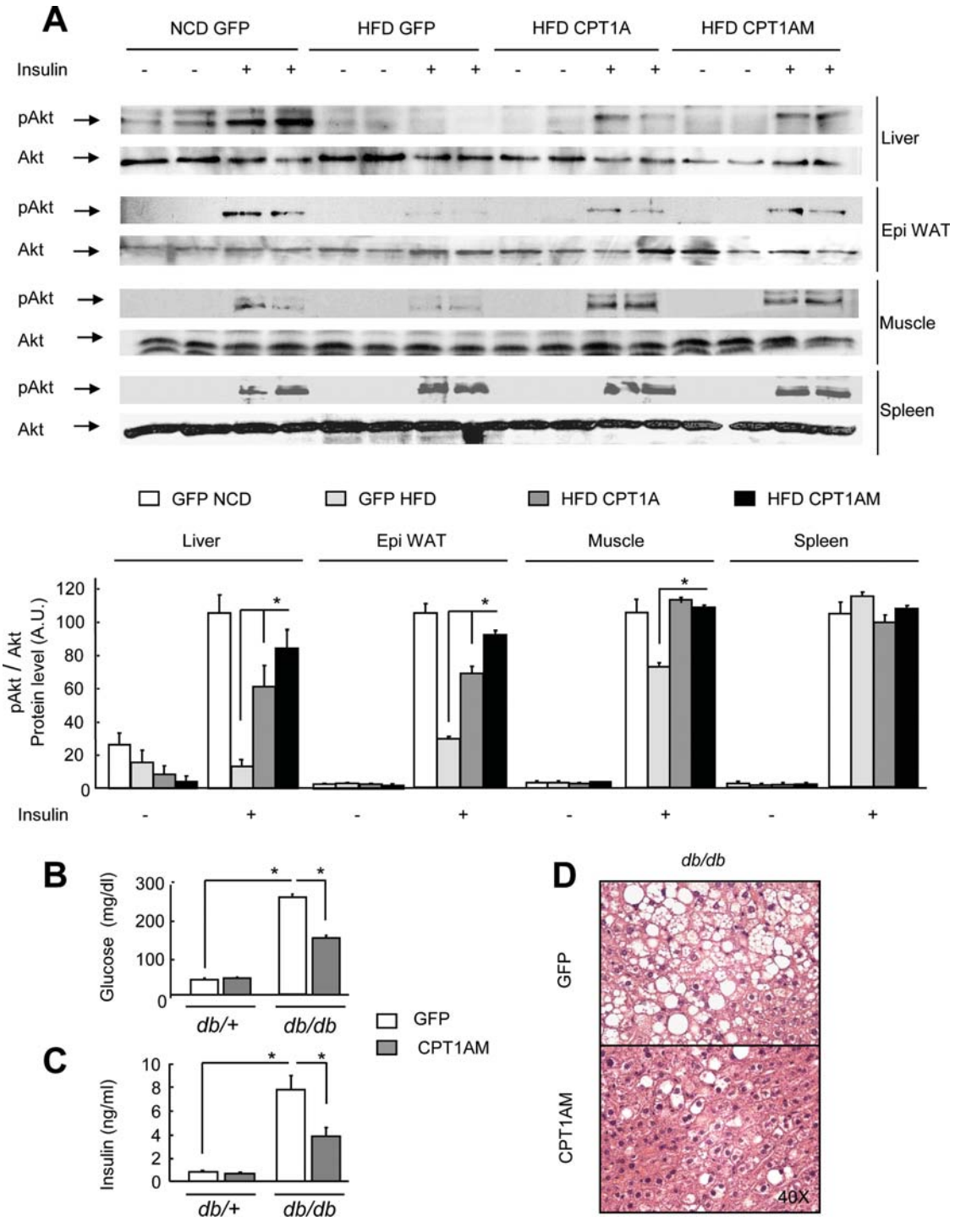

Fig. 7. Improvement of insulin signaling on HFD mice and metabolic parameters in db/db mice. Injected with AAV-CPT1A or AAV-CPT1AM. (A) Insulin signaling in liver and epididymal white adipose tissue (WAT), as indicated by western blotting of insulin-induced AKT phosphorylation (pAKT, Ser473) and quantification of pAKT normalized by total AKT. A.U., arbitrary units. (B) Fasting blood glucose on db/db mice 3 months after AAV injection. (C) Insulin levels on db/db mice 3 months after AAV injection. (D) Liver histological sections from representative db/db mice 3 months after injection with AAV-GFP or AAV-CPT1AM and stained with H&E. n = 8-10. $*P < 0.05$.

and CPT1AM-expressing mice. Importantly, no changes were seen in this case in body weight, hepatic ROS levels, or other hepatic parameters. The present results reinforce the idea that hepatic CPT1A-, and to a greater extent CPT1AM-treatment, is a valid *in vivo* strategy to reduce obesity and improve metabolic parameters without producing undesired alterations on NCD conditions. However, we cannot rule out

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possible side effects produced by hepatic FAO induction for periods longer than those tested in this study.

Several authors have focused on treatments to increase FAO in order to reduce hepatic steatosis, such as liver-specific ACC suppression[5] or hepatic MCD overexpression.[4] They reported a decrease in hepatic TAG content and insulin resistance in obese animals, which is consistent with our findings. However, the contribution of an increase in FAO was difficult to discern because these were short-term studies using approaches that also targeted other metabolic functions. In contrast, a direct increase in FAO through adenovirus-mediated overexpression of CPT1A was reported by O'Doherty and colleagues.[3] The latter study also showed a reduction in hepatic TAG levels, although it was too short to reveal any improvement in insulin sensitivity. Our strategy directly and chronically increased FAO in HFD-treated mice. This led to a decrease in hepatic TAG content and circulating FFA and a consequent improvement in insulin signaling, not only in liver but also in muscle and adipose tissue. Thus, AAV-mediated expression of CPT1A, and to a greater extent CPT1AM, protected mice from HFD-induced whole-animal insulin resistance without altering any of these parameters in NCD control mice.

The lack of pathogenicity of the AAV vectors used and the improvement in hepatic steatosis, serum glucose, and insulin levels observed in the severe obesity developed by genetically obese db/db mice are encouraging results with which to address new challenges related to this gene transfer system. Further studies will be required to elucidate long-term risks which involve both vector and transgene. Uncertainties surrounding gene transfer such as gene integration, the effects of long latencies, and the probability of subtle effects during long-term gene expression must be studied with care. Furthermore, additional caution is needed regarding the feasibility of extrapolating these data to humans, particularly in nonalcoholic fatty liver disease, which is considered a relatively benign disease.

In summary, we report a novel and efficient gene therapy approach to reduce body weight, liver steatosis, fat accumulation in adipose tissue, and obesity-induced diabetes and insulin resistance in rodents. This was achieved by an AAV-mediated, long-term increase in FAO. These results point towards CPT1A as a new potential therapeutic target against obesity-induced disorders.

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