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Am J Physiol Gastrointest Liver Physiol 305: G214–G224, 2013. First published June 6, 2013; doi:10.1152/ajpgi.00102.2013.—CTRP3 is a secreted plasma protein of the C1q family that helps regulate hepatic glucose generation and is downregulated in a diet-induced obese state. However, the role of CTRP3 in regulating lipid metabolism has not been established. Here, we used a transgenic mouse model to address the potential function of CTRP3 in ameliorating high-fat diet-induced metabolic stress. Both transgenic and wild-type mice fed a high-fat diet showed similar body weight gain, food intake, and energy expenditure. Despite similar adiposity to wild-type mice, CTRP3 transgenic mice were strikingly resistant to the development of hepatic steatosis, had reduced serum TNF-α levels, and demonstrated a modest improvement in systemic insulin sensitivity. Additionally, reduced hepatic triglyceride levels were due to decreased expression of enzymes (GPAT, AGPAT, and DGAT) involved in triglyceride synthesis. Importantly, short-term daily administration of recombinant CTRP3 to DIO mice for 5 days was sufficient to improve the fatty liver phenotype, evident as reduced hepatic triglyceride content and expression of triglyceride synthesis genes. Consistent with a direct effect on liver cells, recombinant CTRP3 treatment reduced fatty acid synthesis and neutral lipid accumulation in cultured rat H4IIE hepatocytes. Together, these results establish a novel role for CTRP3 in regulating hepatic lipid metabolism and highlight its protective function and therapeutic potential in attenuating hepatic steatosis.

adipokine; CTRP; C1q/TNF; fatty liver; hepatic steatosis; NAFLD; triglyceride synthesis

HEPATIC STEATOSIS, or fatty liver, results from an imbalance between production and removal of hepatic triglycerides (TAGs) (10). This imbalance can result from excessive alcohol consumption (alcoholic fatty liver disease) or through other means (nonalcoholic fatty liver disease, NAFLD). In NAFLD, elevated hepatic TAG is caused by a combination of excess dietary lipids and de novo fatty acid synthesis (6, 10, 45). Fat oxidation and TAG export (in the form of very low-density lipoprotein, VLDL) aid in removal of hepatic TAGs. NAFLD is one of the primary causes of abnormal liver function (10) and is frequently linked to hepatic insulin resistance and uncontrolled gluconeogenesis in the diabetic state (6, 21, 22, 25, 26, 49). Indeed, up to 70% of clinically obese patients have NAFLD (31). Furthermore, obese patients with NAFLD are at a significantly higher risk of developing obesity-associated comorbidities (e.g., heart disease and Type 2 diabetes) (52). For reasons still poorly understood, a subset of patients with NAFLD will go on to develop NASH (nonalcoholic steatohepatitis) and cirrhosis (10). Despite the prevalence of NAFLD in the general population (28, 50), therapeutic options are limited.

As part of an effort to discover novel secreted metabolic regulators, we recently identified and characterized a family of 15 secreted proteins of the C1q family, designated as C1q/TNF-related proteins (CTRP1–15) (48, 55–57, 60 – 62). Several of these proteins play important and distinct roles in regulating insulin sensitivity and energy balance (11, 42, 43, 54–56, 60, 61). We demonstrated that CTRP3 acts on liver to suppress hepatic glucose output by modulating the expression of gluconeogenic enzymes (43). A cardioprotective function of CTRP3 was recently demonstrated in an animal model of myocardial infarction (67). In addition, several other functions attributable to CTRP3, derived from in vitro studies, have been reported (1–3, 16, 23, 24, 33, 34, 58). In the present study, we sought to address the role of CTRP3 in regulating lipid metabolism and its protective function in a pathophysiological context of high-fat feeding. Using a transgenic (Tg) mouse model, along with short-term recombinant protein supplementation, we established an important and novel role for CTRP3 in regulating hepatic TAG metabolism and highlighted its protective function in attenuating diet-induced hepatic steatosis.

EXPERIMENTAL PROCEDURES

Animals. All animal protocols were approved by the Institutional Animal Care and Use Committee of The Johns Hopkins University School of Medicine. CTRP3 Tg mice (on a C57BL/6 genetic background) and control littermates were housed in polycarbonate cages on a 12-h light-dark photocycle with ad libitum access to water and food. Littermates were used throughout the study as wild-type (WT) controls. Mice were fed a high-fat diet (HFD; 60% kcal derived from fat, Research Diets; D12492) or the isocaloric-matched low-fat diet (LFD; 10% kcal derived from fat, Research Diets; D12450B). Diet was provided for a period of 14 wk, beginning at 4 wk of age. Metabolic parameters and food intake were measured by using the Comprehensive Laboratory Animal Monitoring System (CLAMS) (Columbus Instruments), and body composition was determined by using a whole-body NMR instrument (EchoMRI) as previously described (42). At termination of the study, animals were fasted overnight and euthanized, when tissues were collected, snap frozen in liquid nitrogen, and kept at –80°C until analysis.
Antibodies and chemicals. Mouse monoclonal anti-FLAG M2 antibody was obtained from Sigma. Antibodies that recognize phospho-Akt (Thr-308), phospho-AMPKα (Thr-172), Akt, and AMPKα were obtained from Cell Signaling Technology. Antibody that recognizes actin (sc1616) was obtained from Santa Cruz Biotechnology. Polyclonal rabbit antibody recognizing CTRP3 was obtained from Novus Biologicals (NBP1-02995).

Generation of CTRP3 transgenic mouse line. Carboxy-terminal FLAG epitope (DYKDDDDK)-tagged CTRP3 was cloned into the EcoRI site of pCAGGS vector (40). Expression of Ctrp3 transgene was driven by the ubiquitous CAG promoter, containing a CMV enhancer element with a chicken β-actin promoter. Plasmid construct was digested with SalI and NotI restriction enzymes, and resulting DNA fragments (~3.5 and 2.5 kb) were separated on 1% agarose gel. The ~3.5-kb linear DNA fragment containing the CAG promoter and enhancer, Ctrp3 transgene, and rabbit β-globin polyA adenylation signal was excised from the agarose gel, purified, and verified by DNA sequencing. Pronuclear injections were performed, and several founder lines (on a C57BL/6 genetic background) expressing the Ctrp3 transgene were obtained. One of these mouse lines was maintained and expanded for phenotypic analysis. Tg mice are fertile with no gross abnormality observed.

Mouse serum analysis. Mouse serum samples were collected at times indicated by using microvette CB 300 (Sarstedt). Glucose concentrations were determined at time of blood collection with a glucometer (BD Biosciences). Serum/tissue TAGs (Thermofisher), concentrations were determined at time of blood collection with a glucometer (BD Biosciences). Serum/tissue TAGs (Thermofisher), insulin, tumor necrosis factor-α (TNF-α), and adiponectin (Millipore) were determined with Quantitative PCR analyses were performed on an Applied Biosystems Prism 7500 Sequence Detection System. Samples were analyzed in 25-μl reactions according to the standard protocol provided in the SYBR Green PCR Master Mix (Applied Biosystems). All expression levels were normalized to the corresponding 18 S rRNA levels. Primer sequences can be found in Supplemental Table S1 (Supplemental Material for this article is available online at the Journal website.).

Quantifying the rate of VLDL-triglyceride secretion. To measure hepatic TAG production rate, a separate cohort of HFD-fed mice (Tg and WT littermates) were given an intraperitoneal injection of 1,000 mg/kg poloxamer 407 (Sigma) in saline ~4 h into the light cycle, as described by Millar et al. (35). Poloxamer 407 is an inhibitor of hepatic TAG production and was titrated against the dosing regimen of the glucagon antagonist, GLP-1 analog Liraglutide (Novo Nordisk). Poloxamer 407 was injected 24 h prior to collection.

**Fig. 1.** Generation of CTRP3 Tg mice. A: schematic of Ctrp3 transgenic construct. FLAG-tagged Ctrp3 transgene is driven by a ubiquitous CAG promoter. B: semiquantitative RT-PCR analysis of Ctrp3 transgene expression in mouse tissues; β-actin was included as control. C: immunoblot analysis for the presence of CTRP3-FLAG protein in mouse tissues; β-Actin levels serve as loading control. WT, wild-type; Tg, transgenic.
lipoprotein lipase and it blocks TAG hydrolysis, thus allowing VLDL-TAG molecules to accumulate over time. This process allows for the calculation of hepatic VLDL-triglyceride secretion rates \( (35) \). Serum samples were collected at time \( 0 \) and at 1, 2, 6, and 24 h and analyzed for triglyceride concentration. The TAG production rate was calculated from the differences in plasma TAG levels over a given interval following Poloxamer 407 injection.

**Immunoblot analysis.** Tissue and cell culture homogenates were prepared by using Tissue Protein Extraction buffer (Pierce) supplemented with phosphatase and protease inhibitors (Calbiochem). Protein concentrations were determined by Bradford assay (Thermo Scientific). Ten micrograms of protein from tissue lysates or 1 µl serum were loaded and separated on a 10% Bis-Tris NuPAGE gel (Invitrogen) and transferred to Protran BA8 nitrocellulose membranes (Whatman). Membranes were blocked in 2% nonfat milk and probed with primary and horseradish peroxidase-conjugated secondary antibodies, and chemiluminescence signals were visualized via ECL (GE Healthcare) with Multimage III FluorChem Q (Alpha Innotech). Quantification of signal intensity was performed with Alphaview Software (Alpha Innotech). SeeBlue Plus 2 molecular weight markers (Invitrogen) were used in all immunoblot analysis.

**Protein purification.** Recombinant full-length mouse CTRP3, containing a COOH-terminal FLAG epitope tag, was produced in HEK 293 mammalian cells (GripTite 293; Invitrogen) and purified as described previously \( (43) \). The mammalian expression system ensures proper posttranslational modification and assembly of CTRP3 protein into its correct higher-order structure \( (61) \). Sufficient quantity of recombinant protein was purified from 6 liters of serum-free conditioned media to enable repeated administration into mice. Purified proteins were dialyzed against 20 mM HEPES buffer (pH 8.0) containing 135 mM NaCl in a 10-kDa cutoff Slide-A-Lyzer dialysis cassette (Pierce). Protein concentration was determined by use of a

![Fig. 2. Improved insulin tolerance in Tg mice without changes in other metabolic parameters.](image)

A: no differences in body weight gain over time between WT and Tg male mice fed a high-fat diet (HFD). B: food intake in Tg and WT mice. C: total body mass, fat mass, and lean mass of HFD-fed WT and Tg mice. D–F: indirect calorimetry analysis of oxygen consumption \( (D) \), energy expenditure \( (E) \), respiratory exchange ratio \( (RER \equiv V_{CO2}/V_{O2}; F) \) in HFD-fed Tg and WT mice. G: glucose tolerance test on HFD-fed Tg and WT mice. H: insulin tolerance test on HFD-fed Tg and WT mice. Body weight measurements and glucose and insulin tolerance tests were repeated with multiple cohorts of HFD-fed WT and Tg mice \( (n = 8–10 \text{ per group}) \). Data reported are the results from 1 cohort, with results similar across cohorts. Data are reported as means ± SE of 8–10 mice per group. *\( P < 0.05 \) vs. WT. LF, low-fat diet; HF, High-fat diet. \( V_{O2} \), volume of oxygen consumption; \( V_{CO2} \), volume of carbon dioxide produced; RER, respiratory exchange ratio.
Coomassie Plus protein assay reagent (Thermo Scientific) before samples were aliquoted and stored at −80°C. The purity of recombinant protein was judged to be >95% by Coomassie blue-stained gel.

Cell culture. Rat H4IIE hepatoma cells were maintained in Dulbecco’s modified Eagle medium containing 10% newborn calf serum (Gibco Bio-product). All cell culture experiments were performed in triplicate. Free fatty acid/BSA (bovine serum albumin) conjugates were prepared as described previously (30). Briefly, a 20 mM solution of free fatty acids in 0.01 M NaOH were incubated at 70°C for 30 min, and the fatty acid soaps were then complexed with 5% BSA in PBS at an 8:1 ratio of fatty acid to BSA. Conjugates were administered to cultured cells at concentrations indicated.

In vitro Oil Red O staining. Total neutral lipid content was determined by Oil Red O staining as described by Huang et al. (19). H4IIE hepatocytes were grown to confluence. Cells were then treated overnight with 200 μM palmitate, in the presence of vehicle buffer or CTRP3 (5 μg/ml). Cell were then washed with PBS and fixed for 2 min with 4% formaldehyde. A working solution was prepared from a stock solution of 0.5% Oil Red O isopropyl alcohol, diluted with water (3:2), and filtered through a 0.45-μm filter. The cells were incubated for 1 h at room temperature. After incubation the cells were washed with water and the Oil Red O staining was extracted in isopropanol.

Absorbance was measured at 500 nm with a fluorescent plate reader (model SynergyMx; Biotek).

Fatty acid uptake assay. H4IIE cells were washed twice in PBS and placed in stimulation media (0.1% BSA low-glucose, fatty acid-free DMEM) at 37°C and 5% CO₂ incubator for 2 h. Next, medium was replaced with the same DMEM containing vehicle control, CTRP3 (5 μg/ml), or insulin (50 nM) and incubated overnight. Cells were transferred to a 37°C water bath where 1 μCi/well (in a 24-well format) of [3H]labeled palmitate (dissolved previously for 1 h in the fatty-acid-free DMEM containing 0.1% BSA) was added for either 10, 30, or 60 s. Medium was then aspirated out and cells were washed twice in cold PBS. Cells were lysed in 10% SDS and transferred to a scintillation vial. Radioactive counts were measured and normalized to protein concentration of cell lysate.

Fatty acid synthesis. Fatty acid synthesis was determined via measurement of [3H]acetate incorporation into cells as previously described (44). Briefly, H4IIE hepatocytes were grown to confluence in a 24-well plate. Cells were then treated with vehicle buffer or CTRP3 (5 μg/ml) for 2 h. Next, cold acetic acid (100 mM) and 0.2 μCi/well [3H]acetic acid (American Radiolabeled Chemicals) were added to the media. After 2 h incubation, cells were washed in PBS and lipids were extracted with varying amounts of chloroform-methanol and MgCl₂. Sample was resuspended in a liquid scintillation vial and the amount of [3H]radioactivity was determined by use of a Beckman Coulter counter (model LS6000SC).

Recombinant protein injection. A separate cohort of 4-wk-old C57BL/6 male mice was obtained from the Jackson Laboratory. After 1 wk of acclimatization the mice were placed on an HFD for 12 wk. Mice were fasted for 8 h to obtain initial blood draw (2 h into dark cycle) and then allowed to recover for 72 h with ad libitum access to food. After recovery, initial body weight (considered day 0) was determined. Body weight was measured daily and CTRP3 (2 μg/g body wt) or vehicle buffer was administered daily via intraperitoneal route for the next 5 days. Injections were given at the same time each day (6 h into light cycle). After the fifth injection, food was immediately removed and mice were fasted for 8 h before final blood and tissue collections were performed.

Statistical analyses. Body weights, glucose and insulin tolerance test, and pre/post data from CTRP3 injection experiments were analyzed by a repeated-measures analysis of variance followed by Tukey post hoc analysis. All remaining statistical analyses were performed by a one-way analysis of variance. Statistical analyses were performed with GraphPad Prism 5 statistical software. Statistical significance was accepted at P < 0.05. All data are reported as means ± SE.

RESULTS

Generation of CTRP3 Tg mouse line. We generated a Tg mouse model overexpressing FLAG epitope-tagged CTRP3. Because CTRP3 is a secreted protein and is normally expressed in multiple tissues and cell types in both mouse and human (32, 47, 61), we chose to express the Ctrp3 transgene using a ubiquitous promoter (Fig. 1A). As expected, the Tg mouse line has greater than fivefold higher circulating levels of CTRP3 over baseline serum levels found in wild-type mice (data not shown). At the mRNA level, Ctrp3 transgene was expressed in all tissues examined (Fig. 1B). At the protein level, we detected FLAG-tagged CTRP3 in the liver, heart, muscle, and kidney, but not in brain or adipose tissue (Fig. 1C).

Body weight gain and energy expenditure in response to HFD. The CTRP3 Tg mice developed normally with no obvious phenotype. Body weight gains on LFD (data not shown) and HFD (Fig. 2B) over a period of 14 wk were indistinguishable between Tg and WT mice. No differences were observed in food intake, total fat, or lean body mass between Tg and WT mice fed an LFD (data not shown) or an HFD (Fig. 2, B and C). Oxygen consumption (indicative of basal metabolic rate) and energy expenditure were also similar between HFD-fed Tg and WT mice (Fig. 2, D and E). However, we did observe a modest, but significant, reduction in respiratory exchange ratio (RER) in Tg mice relative to littermate controls (Fig. 2F), indicating a greater utilization of fatty acids as fuel source. No differences in glucose tolerance were observed between Tg and WT mice fed an LFD (data not shown) or an HFD (Fig. 2G), nor were there any differences in the magnitude of insulin secretion between the two groups in response to glucose injection (data not shown). When subjected to insulin tolerance test, however, Tg mice on an HFD clearly demonstrated greater insulin sensitivity relative to WT controls, as indicated by a sustained and significantly greater reduction in blood glucose levels after insulin administration (Fig. 2H).

Fasting serum analysis of HFD-fed Tg and WT mice. Serum levels of hormones and metabolites are tightly linked to metabolic state. We therefore performed blood chemistry analysis on WT and Tg mice. We did not observe any improvements in fasting glucose, insulin, glucagon, NEFA, TAGs, or adiponectin levels following HFD in Tg mice relative to control littermates (Table 1). However, we observed a substantial reduction in serum cholesterol (22%), LDL (31%), and HDL (13%) levels in Tg mice compared with littermate controls (Table 1).

<table>
<thead>
<tr>
<th>Serum Marker</th>
<th>WT</th>
<th>Tg</th>
<th>P Value</th>
</tr>
</thead>
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<tr>
<td>Insulin, mg/ml</td>
<td>1.3 ± 0.18</td>
<td>1.7 ± 0.18</td>
<td>ns</td>
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<tr>
<td>Glucose, mg/dl</td>
<td>109.6 ± 6.5</td>
<td>101.9 ± 10.1</td>
<td>ns</td>
</tr>
<tr>
<td>Glucagon, pM</td>
<td>13.3 ± 0.32</td>
<td>13.8 ± 0.26</td>
<td>ns</td>
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<tr>
<td>Adiponectin, μg/ml</td>
<td>12.5 ± 1.3</td>
<td>13.0 ± 1.3</td>
<td>ns</td>
</tr>
<tr>
<td>TNF-α, pg/ml</td>
<td>6.4 ± 2.0</td>
<td>2.2 ± 0.3</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>Cholesterol (total), mg/dl</td>
<td>138.0 ± 8.6</td>
<td>108.5 ± 6.3</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>LDL, mg/dl</td>
<td>67.2 ± 6.2</td>
<td>46.4 ± 3.9</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>HDL, mg/dl</td>
<td>59.4 ± 2.3</td>
<td>51.5 ± 2.8</td>
<td>P &lt; 0.01</td>
</tr>
<tr>
<td>NEFA, mg/l</td>
<td>0.89 ± 0.06</td>
<td>0.97 ± 0.07</td>
<td>ns</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>41.9 ± 3.6</td>
<td>43.9 ± 3.2</td>
<td>ns</td>
</tr>
</tbody>
</table>

WT, wild-type; Tg, transgenic; LDL, low-density lipoprotein; HDL, high-density lipoprotein; NEFA, nonesterified fatty acids.

Table 1. Blood chemistry analysis of WT and Tg mice
Fig. 3. Reduced hepatic triglyceride content and synthesis in CTRP3 Tg mice. A: representative Tg and WT mouse liver sections stained with Oil Red O. B: quantification of hepatic triglyceride content. C: quantification of mRNA expression of gluconeogenic genes in liver, normalized against 18 S rRNA. D: quantification of mRNA expression of representative fatty acid oxidation genes in liver, normalized against 18 S rRNA. E and F: quantitative immunoblot analysis of liver AMPKα (Thr-172) (E) and Akt (Ser-473) (F) phosphorylation in WT and Tg mice. G: quantification of mRNA expression of enzymes involved in triglyceride synthesis. All data are reported as comparisons between WT and Tg mice on an HFD (n = 8–10 per group). Phosphorylated protein levels were normalized to total protein levels. All data are reported as means ± SE. *p < 0.05 vs. WT.
Low-grade chronic inflammation, reflected in elevated plasma levels of TNF-α, is frequently associated with obesity (17). Strikingly, we also observed a marked reduction (66%) in the circulating levels of TNF-α in Tg mice relative to controls (Table 1).

Reduced expression of lipid synthesis genes and hepatic TAG levels in Tg mice. When liver sections were stained with Oil Red O to detect the presence of neutral lipids, dramatic differences were observed between Tg and WT mice (Fig. 3A), clearly indicating a striking resistance of Tg mice to developing hepatic steatosis in response to HFD. Quantification of hepatic TAG levels confirmed a 38% reduction in TAG levels in Tg mice relative to control littermates (Fig. 3B). Expression of hepatic glucose-6-phosphatase (G6pase), a key gluconeogenic enzyme, was reduced by 90% in Tg mice (Fig. 3C), confirming our previous study based on recombinant CTRP3 protein administration (43). Expression of hepatic Ppar-α, a major transcriptional regulator of fat oxidation genes, was not changed between Tg and WT mice (Fig. 3D), nor were there any differences in the expression of genes directly involved in fat oxidation (e.g., Cpt1a, Acox1, Acad) (Fig. 3D and data not shown). As observed following acute CTRP3 protein administration (43), no significant differences in the phosphorylation levels of AMPKα were detected in the liver of Tg and WT mice (Fig. 3E). In contrast to acute recombinant protein administration (43), when plasma CTRP3 protein was chronically
elevated as in Tg mice, we observed a modest reduction in hepatic Akt phosphorylation (Fig. 3F). Importantly, the expression levels of a number of genes involved in TAG synthesis were substantially reduced in the liver of Tg mice relative to control littermates (Fig. 3G).

**CTRP3 reduces fatty acid synthesis and neutral lipid accumulation in cultured hepatoma cells.** A cell culture system was used to confirm our in vivo findings and to demonstrate that CTRP3 protein directly regulates lipid metabolism in liver cells. When rat H4IIE hepatocytes were coincubated overnight with recombinant CTRP3 protein and 200 μM oleic acid conjugated to BSA to promote lipid loading, the amount of neutral lipids (mainly TAGs) accumulated in cells was significantly reduced (~20%) compared with vehicle-treated controls (Fig. 4A). Whereas the uptake of exogenous fatty acids was not affected by CTRP3 protein treatment (Fig. 4C), de novo fatty acid synthesis, as measured by radiolabeled acetate incorporation, was suppressed (~22%) in H4IIE cells treated with CTRP3 protein (Fig. 4B).

**Measurement of VLDL-TAG export in Tg and WT mice.** To assess the rate and magnitude of VLDL-TAG secretion from the liver, a separate cohort of HFD-fed mice was injected with poloxamer 407, an inhibitor of lipoprotein lipase that blocks VLDL-TAG hydrolysis and clearance (35). Tg mice given poloxamer 407 had a significantly reduced TAG accumulation in the blood (Fig. 5A) and a reduced rate of TAG secretion from the liver (Fig. 5B). Since TAGs are mainly secreted from the liver as VLDL particles, these results suggest that the reduction in hepatic TAG accumulation in Tg mice is indeed due to the suppression of TAG synthesis (Fig. 3) and not caused by increased hepatic VLDL-TAG export.

**Short-term administration of recombinant CTRP3.** Next, we conducted a short-term recombinant protein supplementation study to further ensure that the remarkable phenotype we observed in the liver of Tg mice is directly attributable to elevated plasma CTRP3 levels and not due to potential secondary effects of transgene overexpression. To address this issue, a separate cohort of WT mice was placed on an HFD for 12 wk to induce obesity and the development of fatty liver. DIO mice have similar starting body weights to one another and were given a daily injection of vehicle or recombinant CTRP3 protein (2 μg/g body wt) for 5 consecutive days as outlined (Fig. 6A). Both vehicle- and CTRP3-treated DIO mice lost ~2 g of body weight during the course of the experiment (Fig. 6B). Consistent with our previous findings, in which a single dose of CTRP3 injection acutely reduces blood glucose levels (43), DIO mice that received a 5-day injection also had a 22% reduction in blood glucose levels (Fig. 6C). Strikingly, recombinant protein administration over 5 days resulted in a 43% reduction in hepatic TAGs (vehicle, 155.2 ± 19.4 mg/g vs. CTRP3, 88.6 ± 6.3 mg/g). Serum levels of TAGs and ketones were not different between the two groups of DIO mice (Fig. 6, E and F). Serum ketone levels reflect the extent of hepatic fat oxidation; thus unchanged ketone levels provide further support that hepatic fat oxidation may not be responsible for the reduction of TAG content in the liver of mice injected with recombinant protein. As with the Tg mice, reduced hepatic TAG in CTRP3-injected DIO mice was due to major reduction in the expression of most hepatic enzyme genes involved in TAG synthesis (Fig. 6G).

**DISCUSSION**

In the present study, we provided multiple lines of evidence to establish the role of CTRP3 in regulating hepatic lipid metabolism. Tg mice with elevated plasma levels of CTRP3 are strikingly resistant to the development of HFD-induced hepatic steatosis, independent of other metabolic parameters such as food intake, body weight, adiposity, and energy expenditure. Three possible mechanisms involving production and/or removal of TAG could account for the marked reduction in liver TAG content in Tg mice on an HFD: 1) increased hepatic fat oxidation; 2) increased TAG export from liver in the form of VLDL-TAG particles; 3) decreased synthesis of TAG in liver. Our in vivo and in vitro data suggest that CTRP3-mediated suppression of TAG synthesis is primarily responsible for reduced hepatic TAG content seen in Tg mice.

In liver, TAG is synthesized via the glycerol phosphate pathway (4) through sequential acylation of glycerol-3-phosphate, lysophosphatidic acid, and diacylglycerol by multiple isoforms of GPAT, AGPAT, and DGAT enzymes (51, 66). We show that the expression of these enzymes in liver is significantly suppressed in HFD-fed CTRP3 Tg and wild-type DIO mice administered recombinant CTRP3, thus contributing to reduced hepatic lipid content seen in these animals relative to controls. Remarkably, daily supplementation of recombinant protein for 5 days is sufficient to markedly reduce hepatic TAG levels in wild-type DIO mice, confirming that the improved liver phenotype in Tg mice is due to elevated plasma CTRP3 levels and not a consequence of secondary effects of transgene overexpression. We also noted that serum adiponectin levels were not different between Tg and WT mice, indicating that decreased hepatic TAG content is unlikely due to adiponectin, an adipokine known to alleviate diet-induced hepatic steatosis in mice (64, 65). Adiponectin alleviates hepatic steatosis largely through increasing liver fat oxidation (64); expression of TAG synthesis genes (Gpat, Agpat, and Dgat) were not examined. In contrast, CTRP3 ameliorates fatty liver by reducing hepatic triglyceride synthesis. We cannot completely rule out the possibility that enhanced hepatic fat oxidation may also play a role, as indicated by the lower RER in Tg mice. Even a modest increase in fat oxidation,
Pre-injection blood draw after 8 hr fast

Recovery 8 hr fast

Vehicle
CTRP3

Time (Day)

Body Weight (g)

(8 hr fast)

Vehicle
CTRP3

Blood Glucose (mg/dL)

Before injection
Day 5

Vehicle
CTRP3

Triglycerides (mg/dL)

Ketones (mM)

Liver

Serum

Serum

Triglyceride synthesis

Relative mRNA levels

Vehicle
CTRP3
over time, could potentially result in reduced hepatic TAG content.

We observed a very modest improvement in insulin sensitivity, as judged by insulin but not glucose tolerance test, in HFD-fed CTRP3 Tg mice. This is likely due to improved insulin action in the liver but not skeletal muscle (43). Consistent with this, we did not observe any changes in Akt phosphorylation in the skeletal muscle of Tg mice or WT mice injected with recombinant CTRP3 protein (data not shown). Although the potential function of CTRP3 in skeletal muscle remains unclear, a cardioprotective function of CTRP3 was recently demonstrated in an animal model of myocardial infarction (67), indicating that CTRP3 plays an important role in the heart.

Excessive fat deposition in hepatocytes, a hallmark of steatosis, is frequently associated with hepatic insulin resistance (25, 26, 46, 49). Whether hepatic steatosis causes or is a consequence of insulin resistance is a hotly debated issue (10, 13, 39, 46). Two recent studies using transgenic over-expression of diacylglycerol O-acyltransferase 2 (DGAT2) in mouse liver to alter hepatic lipid content have yielded contradictory results on hepatic insulin sensitivity (21, 37). Also, several other mouse models, with reduced fatty acid synthesis (8), mobilization (5a, 18, 36, 63), or oxidation (38), developed hepatic steatosis without accompanying insulin resistance. Given the very modest improvements in insulin sensitivity seen in the HFD-fed CTRP3 Tg mice compared with littermate controls, it is unclear whether this modest phenotype is due to reduced hepatic lipid content. The mechanistic link between hepatic steatosis and insulin resistance remains to be fully established (39) and is not the focus of present study. Rather, we aim here to establish the role of CTRP3 in regulating lipid metabolism.

We have previously shown that a single injection of recombinant CTRP3 acutely lowered blood glucose levels in WT and genetically obese (ob/ob) mice (43). The CTRP3-mediated suppression of hepatic gluconeogenesis is correlated with the activation of protein kinase B/Akt. In contrast, chronic overexpression of CTRP3 in Tg mice resulted in decreased Akt activation with no change in Peck expression despite a marked suppression of G6Pase expression (Fig. 3, C and F). These results suggest that CTRP3 can inhibit hepatic G6Pase expression independent of Akt signaling whereas the suppression of Peck expression is likely Akt dependent (29). Although chronic overexpression of CTRP3 in Tg mice did not lower fasting blood glucose levels (Table 1), short-term administration of recombinant CTRP3 (one injection per day for 5 days) significantly reduced fasting blood glucose levels in DIO mice (Fig. 6C). The glucose-lowering effect seen in DIO mice is similar to WT and ob/ob mice acutely injected with recombinant CTRP3 (43). Because blood glucose levels are tightly regulated, chronic overexpression of CTRP3 in Tg mice may result in homeostatic compensation to prevent hypoglycemia induced by CTRP3. This may account for the lack of differences in fasting blood glucose levels between WT and Tg mice.

Tg mice fed an HFD have reduced hepatic TAG content compared with WT mice. However, no differences were observed in serum TAG levels between the two groups. The blood chemistry analysis (Table 1) was conducted on sera harvested from overnight-fasted mice. In contrast to the fed state, in which TAG are secreted from liver in the form of VLDL, in the fasted state free fatty acids derived from adipose triglycerides were shunted to the liver to fuel gluconeogenesis and ketones production. Under the fasted state, we did not observe any difference in the steady-state levels of serum TAG between WT and Tg mice.

Interestingly, we observed a decrease in the circulating levels of TNF-α in Tg mice, likely reflecting a dampening of chronic low-grade systemic inflammation associated with high-fat feeding (15, 17). Our in vivo observation is consistent with a previous study demonstrating the ability of recombinant CTRP3 protein to inhibit TNF-α release from primary human macrophages isolated from healthy donors (24). Mice lacking TNF-α or its receptors are protected from obesity-induced insulin resistance (53). Therefore, lower serum levels of TNF-α seen in CTRP3 Tg mice may contribute to the modest improvement in systemic insulin sensitivity.

A reversal or improvement in hepatic steatosis is possible through lifestyle modifications such as reduced energy intake and/or weight loss (41), as well as gastric bypass surgery (31). However, lifestyle changes are often difficult to sustain, necessitating alternative treatment options. One way to reduce liver TAG content is by decreasing TAG synthesis. Previous proof-of-principle studies using siRNA targeting DGAT2 or small molecule inhibitor of GPAT or DGAT1 have demonstrated the feasibility of attenuating hepatic steatosis in rodent (7, 9, 27). In the present study, we show that increasing plasma CTRP3 levels can significantly suppress TAG synthesis through downregulation of TAG synthesis genes (i.e., Agpat, Gpat, and Dgat), thereby improving the fatty liver phenotype in mice without affecting food intake and body weight. This highlights the potential therapeutic value of recombinant CTRP3 protein supplementation in mitigating NAFLD in humans. Given that siRNA or small molecule inhibitor of enzyme often has unintended off-target effects (12, 14, 20), the use of recombinant protein therapy to treat obesity-linked fatty liver may prove to be advantageous.

In sum, we provide novel insights into the metabolic function of CTRP3 and reveal, for the first time, its protective function in liver in response to excess caloric intake. Our data suggest the utility of recombinant CTRP3 as a potential protein therapeutic for treating obesity-associated fatty liver disease.

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DISCLOSURES
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AUTHOR CONTRIBUTIONS
REFERENCES


