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CTRP12 inhibits triglyceride synthesis and export in hepatocytes by suppressing HNF-4α and DGAT2 expression

Stefanie Y. Tan[1,3], **Hannah C. Little**[1], **Dylan C. Sarver**[1], **Paul A. Watkins**[2], **G. William Wong**[1]

[1]Department of Physiology and Center for Metabolism and Obesity Research, Johns Hopkins University School of Medicine, Baltimore, MD, USA

[2]Department of Neurology and Biological Chemistry, Johns Hopkins University School of Medicine, and Hugo W. Moser Research Institute at Kennedy Krieger, Baltimore, MD, USA

[3]Pfizer, 1 Portland St., Cambridge, MA, 02139, USA

Abstract

C1q/TNF-related protein 12 (CTRP12) is an antidiabetic adipokine whose circulating levels are reduced in obesity and diabetes. Although partial and complete loss-of-function mouse models suggest a role for CTRP12 in modulating lipid metabolism and adiposity, its effect on cellular lipid metabolism remains poorly defined. Here, we demonstrate a direct action of CTRP12 in regulating lipid synthesis and secretion. In hepatoma cells and primary mouse hepatocytes, CTRP12 treatment inhibits triglyceride synthesis by suppressing glycerophosphate acyltransferase (GPAT) and diacylglycerol acyltransferase (DGAT) expression. CTRP12 treatment also downregulates the expression of hepatocyte nuclear factor-4α (HNF-4α) and its target gene microsomal triglyceride transfer protein (MTTP), leading to reduced very-low-density lipoprotein (VLDL)-triglyceride export from hepatocytes. Consistent with the *in vitro* findings, overexpressing CTRP12 lowers fasting and postprandial serum triglyceride levels in mice. These results underscore the important function of CTRP12 in lipid metabolism in hepatocytes.

Keywords

adipokine; adipolin; CTRP12; hepatocyte; lipid metabolism; triglyceride synthesis

Correspondence: G. W. Wong, Department of Physiology and Center for Metabolism and Obesity Research, Johns Hopkins University School of Medicine, Baltimore, MD 21205, USA, Tel: 410 502 4862, gwwong@jhmi.edu.

Author contribution

SYT and GWW contributed to the experimental design. SYT, HCL, and DCS performed the experiments. PAW provided analytical and intellectual input. SYT and GWW analyzed and interpreted the data, and SYT and GWW wrote the paper.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. CTRP12 treatment has no effect on the expression of enzymes involved in de novo lipogenesis in H4IIE hepatoma cells.

Fig. S2. CTRP12 does not affect fatty acid uptake in H4IIE hepatoma cells.

Fig. S3. CTRP12 treatment has no effect on Hnf-1α expression in H4IIE hepatoma cells.

Fig. S4. CTRP12 treatment has no effect on the expression of enzymes involved in de novo lipogenesis in primary mouse hepatocytes.

Fig. S5. Expression of triglyceride synthesis genes in the liver of CTRP12 overexpression and deficient mice.

Table S1. Mouse and rat primer sequences used in real-time PCR analyses.

Energy homeostasis is controlled by central and peripheral mechanisms [1]. Integration of nutritional signals and the consequent adaptive response to changes in physiological states in the periphery are, in large part, orchestrated by secreted hormones [2,3]. In the context of metabolism, dysregulated organ crosstalk frequently leads to aberrant blood glucose and/or lipid levels, and this contributes to the development of type 2 diabetes mellitus (T2DM) and dyslipidemia [4,5]. Among the secretory proteins with metabolic functions, adipose-derived adipokines (e.g., adiponectin, leptin, omentin, resistin, RBP4) have been the focus of intense study [2,6]. These proteins either act directly on metabolic tissues (adipose, liver, skeletal muscle) via paracrine or endocrine mechanisms, or act indirectly through their actions on immune cells, to modulate local and systemic metabolism [2,3,6–8].

Our efforts to identify novel secreted metabolic regulators have led to the discovery of a highly conserved family of plasma proteins of the C1q family, referred to as C1q/TNF-related proteins (CTRP1–15) [9–17]. Genetic mouse models and recombinant protein infusion *in vivo* have provided physiological evidence for CTRPs in regulating sugar and fat metabolism, as well as food intake and insulin sensitivity [15,16,18–26].

CTRP12 was discovered based on shared sequence homology with other CTRP family members and the presence of the signature C1q domain [12]. The transcript is encoded by the *FAM132A* gene. It was also identified as an adipose-enriched transcript (designated adipolin) downregulated in obesity [27]. The expression of human *CTRP12* is highly enriched in adipose tissue; in mice, however, it is more broadly expressed [12,27]. In animal models of diet-induced obesity and diabetes, the expression of *Ctrp12* transcript in adipose tissue is markedly downregulated [12,27]. In cultured adipocytes, inflammatory cytokines such as TNF-α can suppress *Ctrp12* expression through downregulating Krupple-like factor 15 (KLF15), a transcriptional regulator of *Ctrp12* gene [28]. In contrast, *Ctrp12* expression is upregulated in KLF3 knockout mice with enhanced insulin sensitivity [29].

The physiological relevance of CTRP12 in humans is underscored by recent studies. For instance, in healthy lean adults, insulin infusion elevates plasma CTRP12 levels [30], and its expression and secretion from human adipose tissue explants are augmented by antidiabetic drugs such as rosiglitazone and metformin [30,31]. Conversely, circulating levels of CTRP12 are reduced in patients with type 2 diabetes [32], polycystic ovarian syndrome (PCOS) [33], and coronary artery disease [34]. The plasma level of CTRP12 in women with PCOS can be elevated by metformin administration [35]. Interestingly, humans with a deletion spanning chromosome 1p36.33, which includes the *CTRP12/FAM132A* gene locus, develop hyperphagia and obesity [36–39].

Initial gain-of-function studies in mice have revealed antidiabetic [12] and anti-inflammatory [27] activities of CTRP12. Recent studies also implicated a protective role of CTRP12 in the cardiovascular system [40,41]. In the context of metabolism, CTRP12 acts through insulin-dependent and insulin-independent pathways to control glucose metabolism in liver and adipose tissue [12]. In healthy lean mice, as well as in severely obese and insulin-resistant *ob/ob* (leptin-deficient) mice, increasing plasma level of CTRP12 via adenoviral-mediated overexpression improved insulin action and lowered blood glucose [12,27]. In cell culture models, CTRP12 treatment stimulates glucose uptake in adipocytes and suppresses

gluconeogenesis in hepatocytes [12]. Mice with a partial (+/−) or complete (−/−) deletion of the *Ctrp12* gene exhibit sexually dimorphic phenotypes with regard to adiposity and lipid profiles [42,43]. Ablating the *Ctrp12* gene exacerbates obesity and insulin resistance in male mice fed a high-fat diet; in contrast, female mice lacking CTRP12 have lower weight gain when challenged with a high-fat diet [42].

While the physiological function of CTRP12 has been established in various genetic mouse models, its mechanism of action remains incompletely understood. Importantly, the direct action of CTRP12 on cellular lipid metabolism has not been demonstrated. Using cell model systems, we provided evidence for a direct action of CTRP12 in regulating triglyceride synthesis and export from hepatocytes. Our *in vivo* studies further support our *in vitro* findings.

Materials and methods

Recombinant protein

Production and purification of C-terminal FLAG-tagged recombinant mouse CTRP12 were performed in a mammalian expression system as previously described [12]. Importantly, this enabled the generation of recombinant CTRP12 with all the appropriate post-translational modifications such as N-linked glycosylation and higher-order structure formation [12]. In brief, HEK 293 GripTite™ cells (Invitrogen) were transfected with plasmids containing CTRP12 cDNA constructs using polyethylenimine, MW 40 000 (Polysciences, Inc., Warrington, PA, USA). At 36–48 h post-transfection, media were replaced with serum-free Opti-MEM (Invitrogen, Carlsbad, CA, USA) supplemented with 100 $\mu g \cdot mL^{-1}$ vitamin C. Serum-free conditioned media were collected and subjected to affinity chromatography using the Anti-FLAG® M2 Affinity Gel (Sigma-Aldrich, St. Louis, MO, USA). Eluted protein was concentrated and dialyzed in HEPES buffer (20 mM HEPES, 135 mM NaCl, pH 8.0) using a 10-kDa cutoff Amicon Pro centrifugal unit (EMD Millipore, Burlington, MA, USA). Based on our previous study [12], 10 $\mu g\ mL^{-1}$ of recombinant CTRP12 was used in all our current *in vitro* assays.

Cell culture

HEK 293 GripTite™ cells were cultured in DMEM (Invitrogen) containing 10% FBS supplemented with 2 mM L-glutamine, 100 $units \cdot mL^{-1}$ penicillin, and 100 $\mu g\ mL^{-1}$ streptomycin. Rat H4IIE hepatoma cells were cultured in DMEM containing 5 mM glucose, 10% FBS, and 1% antibiotics (Pen/Strep).

Primary hepatocyte isolation and culture

Primary mouse hepatocytes were isolated from 14- to 17-week-old C57BL/6J mice based on a previously published protocol [44]. Pure hepatocytes were resuspended in 15-mL complete culture medium [Media 199 with 10% fetal bovine serum (Gibco, Gaithersburg, MD, USA and Atlanta Biologicals, Minneapolis, MN, USA, respectively) and 1% Pen/Strep], and the number of live cells and dead cells counted with a hemacytometer following Trypan blue staining. Cells isolated with a viability of 93% or greater were used. 1.25×10^5 *live* cells

were plated per well in 24-well plates precoated with collagen I (Gibco) in 500 μL complete culture medium. Cells were incubated for 18–24 h before treatment.

Mice

Wild-type C57BL/6J male mice were purchased from the Jackson Laboratory. The mice were maintained on a standard laboratory chow (Harlan Teklad, Indianapolis, IN, USA), had free access to food and water, and were housed in plastic cages on a 12-h light/12-h dark photocycle. All of the animal experiments were approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

Adenovirus preparation and infection

Type 5 adenovirus encoding control GFP or mouse CTRP12 was generated by Vector Biolabs. Adenovirus encoding either GFP or CTRP12 was injected via the retro-orbital sinus into male mice (8–9 weeks of age) at a dose of 1×10^9 infectious particles per mouse.

Fasting-refeeding experiments

Mice were fasted overnight on day 7 postadenovirus injections, and fasting sera were obtained via tail bleed. Fasting blood glucose was measured using a glucometer. Standard laboratory chow (Harland) was then re-introduced, and mice were allowed to feed ad libitum for 3 h. Blood sera were obtained at 90 min and 3 h postrefeeding via tail bleed and terminal retro-orbital bleed, respectively.

Western blot analysis

Cell lysates were prepared by washing cells twice with cold PBS and lysed in whole-cell lysis buffer (20 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM sodium EDTA, 0.5% NP-40, 10% glycerol). Total cell protein was quantified using a Bradford assay and mixed with sample loading buffer (50 mM Tris, 2% SDS, 1% β-ME, 6% glycerol, 0.01% bromophenol blue), sonicated, and incubated at 100°C for 10 min before subjected to SDS/PAGE analysis. 12.5–20 μg of total protein was loaded per well. Protein was transferred onto nitrocellulose or PVDF membranes and blocked in PBS containing 0.2% Tween 20 and 5% nonfat milk for 1 h, and then probed overnight with the following antibodies: acetyl-CoA carboxylase (ACC; Cell Signaling Technology, Danvers, MA, USA), fatty acid synthase (FAS; BD Transduction Laboratories, San Jose, CA, USA), stearoyl-CoA decarboxylase 1 (SCD1; Santa Cruz Biotechnology, Dallas, TX, USA), glycerol-3-phosphate acyltransferase 1 (GPAT1; Thermo Scientific), diacylglycerol acyltransferase 2 (DGAT2; Abcam, Cambridge, MA, USA), β-tubulin (Abcam), microsomal triglyceride transfer protein (MTTP; BD Transduction Laboratories), heat shock cognate protein 70 (Hsc70; Santa Cruz Biotechnology), and hepatocyte nuclear factor 4α (HNF-4α; R&D Systems). Anti-mouse IgG, anti-rabbit IgG (Cell Signaling Technology), and anti-goat IgG (Millipore) secondary antibodies conjugated to HRP were used to recognize the primary antibodies. Western blot membranes were developed with HRP substrate ECL (GE Healthcare, Marlborough, MA, USA) and visualized with MultiImage III FluorChem® Q (Alpha Innotech, San Leandro, CA, USA). Quantifications of signal intensity were performed using ALPHAVIEW Software (Alpha Innotech).

Quantitative real-time PCR (qPCR) analysis

Total RNA was isolated from mouse liver (Ad-GFP, Ad-CTRP12, *Ctrp12* wild-type, *Ctrp12* knockout), as well as cultured cells treated with recombinant CTRP12 at the indicated time point using TRIzol® and reverse-transcribed using GoScript Reverse Transcriptase (Promega, Madison, WI, USA). The quantitative real-time PCR analyses were performed on a CFX Connect system (Bio-Rad Laboratories, Hercules, CA). cDNA samples (10 ng) were analyzed in 10 μL reactions according to the standard protocol provided in the SYBR® Green iTaq Master Mix (Bio-Rad). Results were analyzed using the $2^{-\Delta\Delta Ct}$ method [45]. All expression levels were normalized to corresponding *β-actin* RNA levels. Rat and mouse primers used are listed in Table S1.

^{3}H-Acetate and ^{14}C-oleate incorporation assays

Lipogenesis was studied using ^{3}H-acetate and ^{14}C-oleate incorporation assays. ^{3}H-acetate and ^{14}C-oleate incorporation was performed in H4IIE cells and primary mouse hepatocytes. Cells were plated in a 24-well plate, grown to 90% confluence, and treated for 16 h with control vehicle buffer or CTRP12 (10 μg·mL^{-1}), before labeling with 10 μCi of ^{3}H-acetate or 0.1 μCi ^{14}C-oleic acid per well. Incorporation of radiolabeled acetate or oleate was allowed to proceed for 2 h in H4IIE cells or primary hepatocytes, unless otherwise stated. Labeling was terminated by removing the labeled media. Cells were washed three times with cold PBS, and total cellular lipids extracted with the Folch method [46]. Incorporation was quantified by scintillation counting of extracted lipids.

Measurement of secreted triglycerides

Triglycerides (TGs) secreted into culture medium by H4IIE cells and primary hepatocytes were measured using the triglyceride and free glycerol Infinity reagent (Thermo Scientific) according to the manufacturer's instructions. Secreted TGs were also measured from culture medium of H4IIE cells following a ^{3}H-acetate incorporation assay. Media were collected after termination of the assay, the total lipid fraction was collected using the Folch method[46], and secreted lipids containing radiolabeled acetate were quantified by scintillation counting. TGs in serum were measured using Infinity kits (Thermo Scientific) according to the manufacturer's instructions.

^{14}C-oleate uptake assay

Fatty acid uptake into H4IIE cells was measured as previously described [15]. Briefly, cells were treated overnight with 10 μg·mL^{-1} CTRP12 or vehicle control buffer, and then, ^{14}C-oleate uptake was allowed to proceed for the stated times. Fatty acid uptake was terminated by the addition of ice-cold stop buffer (1 mM $MgCl_2$, 1.2 mM $CaCl_2$, 0.1% fatty acid-free BSA, 200 μM phloretin). Cells were washed 3 times in ice-cold PBS, lysed in whole-cell lysis buffer, and radiolabeled fatty acid uptake was quantified by scintillation counting of cell lysates.

Results

CTRP12 suppresses lipogenesis in rat H4IIE hepatoma cells

To investigate the effects of CTRP12 on lipogenesis, we examined *de novo* fatty acid synthesis and the esterification of fatty acids into TG in H4IIE hepatoma cells. This widely used rat liver cell line responds physiologically to the effects of insulin on lipid and glucose metabolism [47–56]. Importantly, the *in vivo* effects of CTRP12 on *de novo* glucose production are recapitulated in H4IIE cells [12]. Here, we show that overnight treatment of H4IIE cells with recombinant CTRP12 significantly suppressed lipid synthesis, as measured by the incorporation of both radiolabeled acetate and oleate into the total cellular lipid fraction (Fig. 1A,B).

CTRP12 downregulates the expression of enzymes involved in TG synthesis

To explore the underlying basis for the suppression of acetate and oleate incorporation, we examined the effects of CTRP12 administration on the expression of enzymes involved in fatty acid and TG synthesis. Expression of *de novo* fatty acid synthesis genes—acetyl-CoA carboxylase (*Acc1*), fatty acid synthase (*Fasn*), and stearoyl-CoA desaturase (*Scd1*)—was not affected by CTRP12 treatment (Fig. S1A). Total ACC1, FAS, and SCD1 protein levels also remained unchanged by CTRP12 treatment (Fig. S1B). In contrast, the expression of TG synthesis genes—glycerophosphate acyltransferase (*Gpat1*), *Gpat4*, and diacylglycerol acyltransferase (*Dgat2*)—was significantly downregulated in H4IIE cells treated with CTRP12 (Fig. 1C). In parallel, we observed a corresponding reduction in GPAT1 and DGAT2 protein levels in H4IIE cells treated with CTRP12 (Fig. 1D,E). A possible reason for the observed suppression in fatty acid incorporation into the total lipid fraction could be decreased fatty acid uptake into cells, resulting in a smaller pool of cellular fatty acids available for lipid synthesis. To rule this out, we examined the effects of CTRP12 on oleic acid uptake into H4IIE cells. No difference in ^{14}C-oleic acid uptake was observed in H4IIE cells treated with vehicle control or CTRP12 (Fig. S2).

CTRP12 suppresses TG export from H4IIE hepatoma cells

TGs are packaged and exported from hepatocytes as very-low-density lipoprotein (VLDL) particles, and the amount of VLDL secreted is positively correlated with hepatic TG content and its synthesis [57]. We reasoned that a reduction in TG synthesis would likely result in a corresponding decrease in triglyceride export from H4IIE cells. To test this, control H4IIE cells and those treated overnight with CTRP12 were labeled with ^{3}H-acetate for 1 or 2 h, and the amount of radioactivity in the neutral lipid fraction of the media was quantified. Compared to vehicle control, CTRP12 treatment markedly suppressed the secretion of radiolabeled neutral lipids (mainly TG) from H4IIE cells (Fig. 2A), suggesting that CTRP12 reduced TG synthesis from endogenously synthesized fatty acids derived from ^{3}H-acetate, and their subsequent export. To specifically quantify the amount of TG secreted by H4IIE cells into the culture medium, we measured TG levels in media from cells treated overnight with control buffer, insulin, or CTRP12. As expected, treatment with insulin, known to suppress hepatic TG secretion [58–60], significantly reduced the amount of TG detected in the media (Fig. 2B). Similarly, CTRP12 treatment significantly reduced the amount of TGs that were secreted into the conditioned media of H4IIE cells (Fig. 2B).

CTRP12 downregulates microsomal triglycerol transfer protein expression

Each VLDL particle consists of neutral lipids (mostly TG and some cholesteryl esters) and a single apolipoprotein B (ApoB) [61]. Loading of TG onto nascent ApoB by microsomal triglyceride transfer protein (MTTP) in the endoplasmic reticulum (ER) initiates the lipidation of apolipoprotein particle and VLDL assembly [62,63]. MTTP expression is regulated at the transcriptional level [64]. To uncover the mechanism by which CTRP12 suppresses TG secretion, we first examined the effects of CTRP12 treatment on the expression of MTTP, an essential protein for VLDL assembly and secretion in hepatocytes [62,65]. CTRP12 treatment resulted in a significant downregulation of MTTP mRNA and protein expression in H4IIE cells (Fig. 2C,D).

CTRP12 suppresses the expression of HNF-4α

Expression of MTTP is transcriptionally regulated by hepatocyte nuclear factor 4α (HNF-4α), a member of the nuclear receptor superfamily that controls many critical genes involved in hepatic lipid metabolism [66]. Conditional deletion of *Hnf-4α* markedly reduces serum cholesterol and TG due to suppressed expression of MTTP [67]. Thus, we asked whether CTRP12-induced reduction in MTTP expression is mediated by HNF-4α. Treatment of H4IIE cells with CTRP12 potently suppressed the mRNA and protein expression of HNF-4α (Fig. 2E,F). In contrast to *Hnf-4α*, CTRP12 treatment did not reduce the mRNA expression of *Hnf-1α* (Fig. S3).

CTRP12 treatment suppresses TG synthesis and export in primary mouse hepatocytes

To confirm our results in a more physiological model, we examined TG export in primary hepatocytes isolated from wild-type mice. Consistent with our observations in H4IIE cells, treatment of primary mouse hepatocytes with recombinant CTRP12 suppressed TG secretion into the culture medium (Fig. 3A). Also consistent with our observations in H4IIE cells, mRNA and protein levels of *de novo* fatty acid synthesis genes in primary mouse hepatocytes were unchanged by CTRP12 treatment (Fig. S4), while genes (*Gpat1* and *Gpat4*) and protein (DGAT2) involved in TG synthesis were reduced (Fig. 3B,C). Expression of MTTP mRNA and protein was similarly suppressed in primary mouse hepatocytes by CTRP12 (Fig. 3D,E). We next addressed whether CTRP12-mediated downregulation of these genes was linked to HNF-4α in primary hepatocytes. Parallel to what we observed in H4IIE cells, CTRP12 treatment also suppressed mRNA and protein expression of HNF-4α in primary hepatocytes (Fig. 3F,G).

CTRP12 overexpression in mice lowers fasted and postprandial serum triglyceride

To further assess whether our *in vitro* findings hold true *in vivo*, we overexpressed CTRP12 in mice via adenovirus delivery. We have previously shown that adenoviral-mediated overexpression resulted in maximally threefold increase in plasma CTRP12 over physiologic baseline in WT mice [12]; rise in plasma CTRP12 peaks on day 5 and declines back to baseline level by day 10 postinfection. Consistent with previous report [12], CTRP12 overexpression (Ad-CTRP12) lowered fasting blood glucose levels in mice relative to animals expressing control GFP (Ad-GFP) (Fig. 4A). Corroborating our findings in hepatoma cells and primary hepatocytes, fasting serum TG levels were also significantly

reduced in Ad-CTRP12 mice compared with Ad-GFP controls on day 5 postinfection (Fig. 4B), at a time point when CTRP12 levels were presumed to be at its highest level. When the mice were refed following an overnight fast, serum TG levels were further suppressed in Ad-CTRP12 mice compared with control Ad-GFP mice at 90 min postrefeeding (Fig. 4C). We measured hepatic mRNA expression of *Hnf-4α*, *Gpat1*, *Gpat4*, *Dgat2*, and *Mttp* on day 10 when the animals were euthanized. At this time point when plasma levels of CTRP12 were presumed to have declined back to baseline, we did not observe any significant differences in hepatic expression of *Hnf-4α*, *Gpat1*, *Gpat4*, *Dgat2*, or *Mttp* between Ad-GFP and Ad-CTRP12 mice (Fig. S5). Although we expected the transcript levels of these same genes to be upregulated in *Ctrp12* knockout (KO) mice, no differences in hepatic expression of *Hnf-4α*, *Gpat1*, *Gpat4*, *Dgat2*, or *Mttp* were observed between genotypes (Fig. S5).

Discussion

Our data highlight the regulatory role of CTRP12 in several aspects of lipid metabolism. Using rat H4IIE hepatoma cells and primary mouse hepatocytes as *in vitro* model systems, we show that CTRP12 acts directly on liver cells to reduce lipid synthesis. CTRP12 treatment suppressed both acetate and oleate incorporation into total lipids, indicating reduced *de novo* lipogenesis, and TG synthesis, from exogenous fatty acids. This effect is not a result of impaired lipid uptake, as CTRP12 treatment did not change the rate of fatty acid uptake into cells. CTRP12 treatment also did not alter the mRNA and protein expression of key *de novo* lipogenic enzymes such as ACC1, FAS, and SCD1. In contrast, CTRP12 treatment significantly reduced the mRNA and protein expression of enzymes—GPAT1, GPAT4, and DGAT2—involved in TG synthesis through the glycerol phosphate pathway [68]. These results suggest that reduced lipogenesis, as determined by radiolabeled acetate and oleate incorporation into total lipids, stemmed predominantly from reduction in TG synthesis.

In hepatocytes, TG synthesis and secretion (as VLDL) are generally coupled [57,62]. This prompted us to assess whether TG export from liver cells is also suppressed by CTRP12. Not surprisingly, since CTRP12 suppressed TG synthesis, it also significantly reduced TG secretion from H4IIE hepatoma cells and primary mouse hepatocytes. To be secreted, TG needs to be loaded onto nascent ApoB in the ER to initiate VLDL assembly, a process requiring MTTP [61,62,69]. When we examined the expression of MTTP in response to CTRP12 treatment, we found that MTTP mRNA and protein levels were significantly reduced. One possible mechanism regulating MTTP expression is via the liver-enriched nuclear receptor, HNF-4α (nuclear receptor 2A1), known to be a critical transcriptional regulator of many key genes involved in hepatic lipid metabolism [66]. Among the direct transcriptional targets of HNF-4α is *Mttp*. Liver-specific deletion of HNF-4α in mice strikingly suppresses hepatic expression of MTTP, leading to a marked reduction in circulating TG levels [67]. We show that CTRP12 treatment potently suppressed HNF-4α mRNA and protein levels in both H4IIE hepatoma and primary mouse hepatocytes. CTRP12-mediated reduction in TG export in hepatocytes is likely due to a combined effect of decreased TG synthesis and its incorporation into VLDL. The fact that CTRP12 downregulates MTTP expression at the level of transcription is consistent with the previous studies indicating transcriptional control as the major mode of MTTP regulation in

hepatocytes [64,70–72]. These results suggest that CTRP12 likely regulates the steady-state expression level of MTTP in hepatocytes via HNF-4α.

To address the physiological relevance of our *in vitro* findings, we extended our studies in mice. Based on our cell model studies, we anticipated that acute CTRP12 overexpression would reduce circulating TG levels *in vivo*. Indeed, in wild-type mice injected with adenovirus encoding CTRP12, fasting and postprandial serum TG levels were significantly lower when compared to animals expressing GFP control. Circulating TG levels are decreased in Ad-GFP and Ad-CTRP12 mice at 90 min following postrefeeding; this may be due to the postprandial suppression of free fatty acid flux into the liver from adipose tissue lipolysis. These *in vivo* observations are in line with the suppressive action of CTRP12 on TG secretion seen in H4IIE hepatoma cells and primary hepatocytes.

In sum, our studies establish a direct regulatory role for CTRP12 in modulating lipid synthesis and export in hepatocytes. Furthermore, we demonstrate that short-term overexpression of CTRP12 in mice is sufficient to modulate systemic lipid metabolism. Given that hepatic VLDL secretion and other aspects of lipoprotein metabolism are dysregulated in the pathophysiological states of obesity, insulin resistance, and T2DM [57], our studies may shed light on possible crosstalk between adipose-derived hormones (e.g., CTRP12) and hepatic lipid metabolism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Abbreviations

DGAT	diacylglycerol acyltransferase
ER	endoplasmic reticulum
GFP	green fluorescent protein
GPAT	glycerophosphate acyltransferase
HNF-4α	hepatocyte nuclear factor-4α
MTTP	microsomal triglyceride transfer protein
PCOS	polycystic ovarian syndrome
T2DM	type 2 diabetes mellitus
TGs	triglycerides
VLDL	very-low-density lipoprotein

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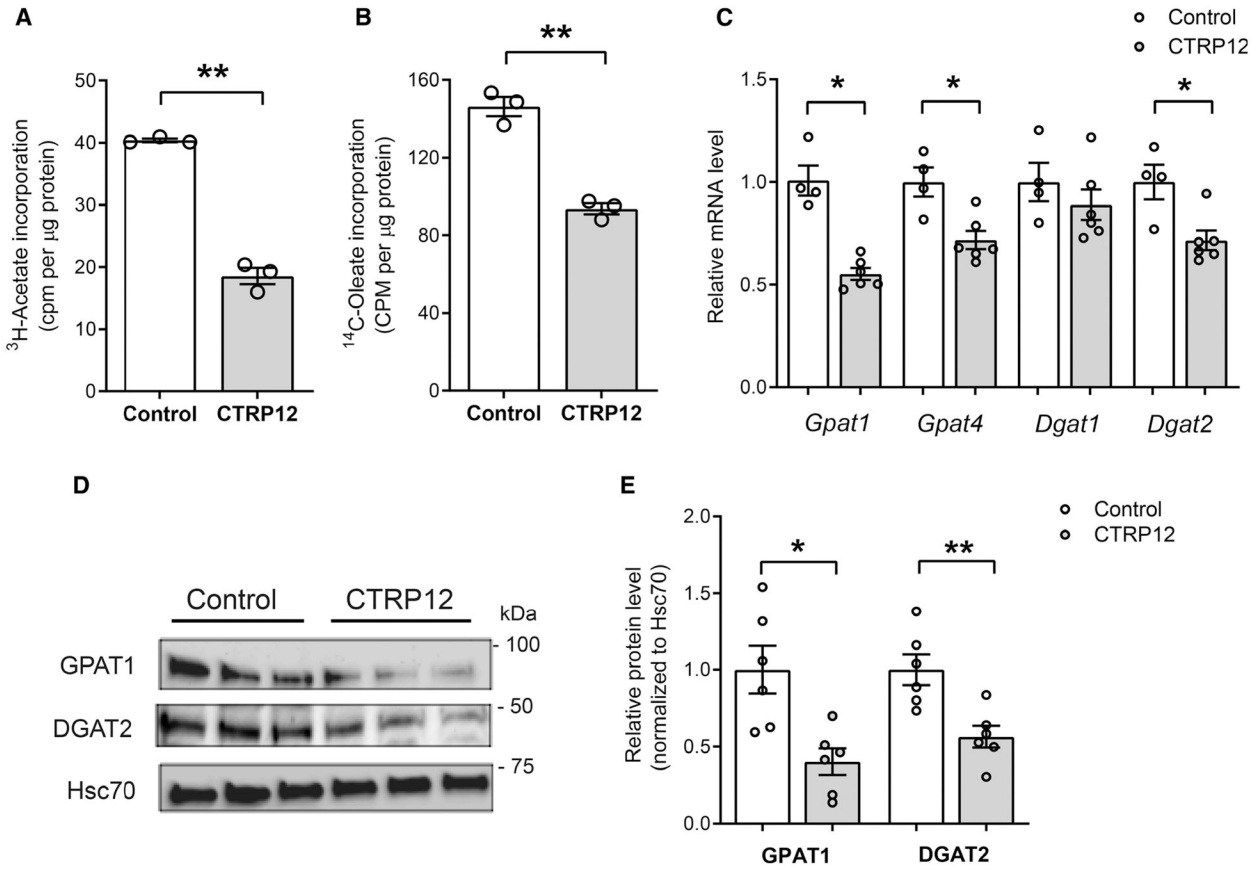

Fig. 1.

CTRP12 inhibits triglyceride synthesis in rat hepatoma cells by suppressing GPAT1 and DGAT2 expression. (A) ^{3}H-acetate incorporation and (B) ^{14}C-oleate incorporation into total lipid fraction in H4IIE cells (n = 3 per group) treated overnight (16 h) with control buffer or purified recombinant CTRP12 (10 µg·mL^{-1}). Radiolabeled acetate or oleate utilization is expressed as CPM incorporated into the total lipid fraction normalized to total protein content. (C) Relative mRNA expression of triglyceride synthesis genes [glycerol phosphate acyltransferase (*Gpat*), diacylglycerol acyltransferase (*Dgat*)] in H4IIE cells (n = 4–6) treated for 24 h with control buffer or recombinant CTRP12 (10 µg·mL^{-1}). (D) Western blot analysis of GPAT1 and DGAT2 protein levels 24 h post-treatment. (E) Quantification of GPAT1 and DGAT2 as shown in D (n = 6). *, P < 0.05; **, P < 0.01. Two-tailed Student's t-tests were performed for all panels.

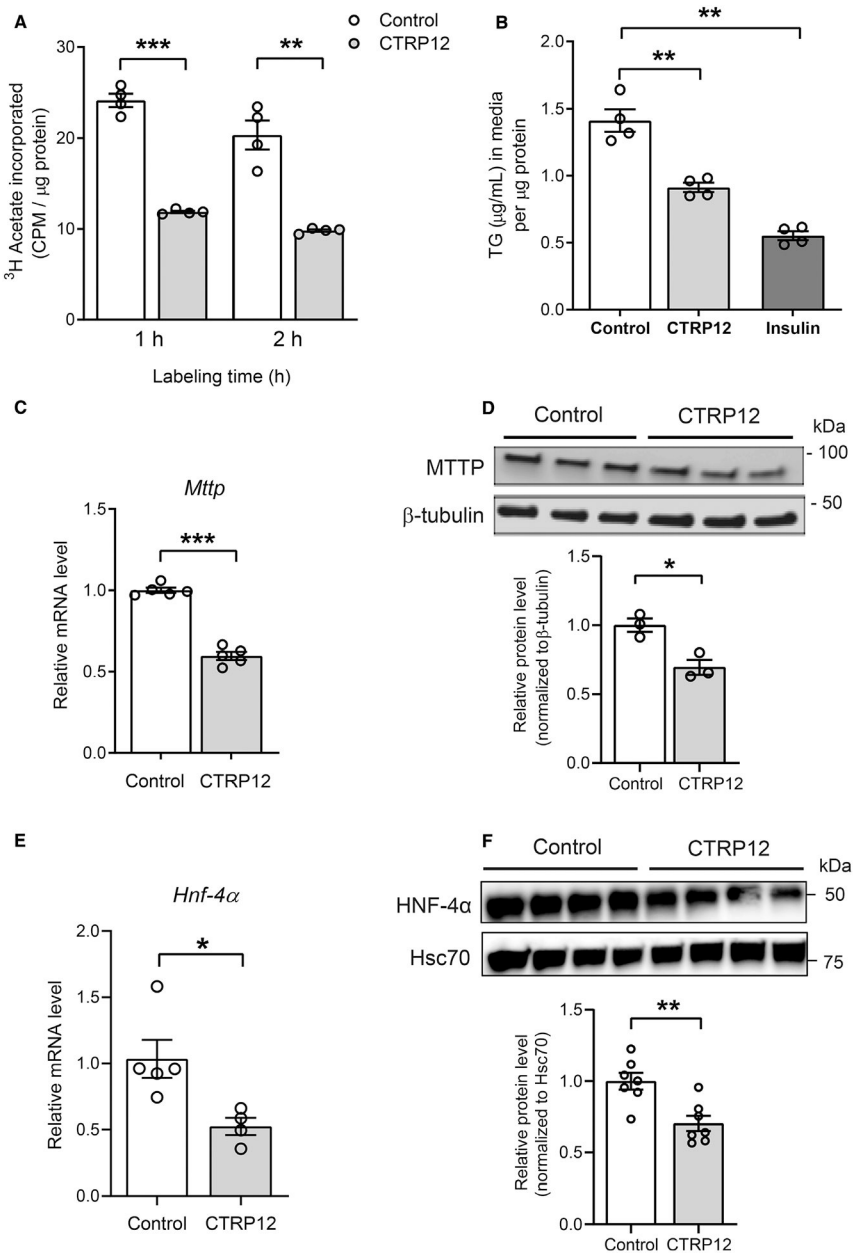

Fig. 2.

CTRP12 reduces triglyceride export from rat hepatoma cells by downregulating the expression of MTTP and HNF-4α expression. (A) Radiolabeled neutral lipids secreted into the conditioned media of H4IIE cells ($n = 4$) treated for 16 h with control buffer or recombinant CTRP12, and then labeled with ^{3}H-acetic acid for 1 or 2 h. (B) Triglyceride (TG) exported into the conditioned media of H4IIE cells ($n = 4$ per group) treated for 24 h with control buffer, recombinant CTRP12 (10 μg·mL^{-1}), or insulin (20 nM). All triglyceride concentrations were normalized to total protein content in the cells. (C) Relative mRNA expression of microsomal triglyceride transfer protein (*Mttp*) in H4IIE cells ($n = 5$) treated with control buffer or recombinant CTRP12 (10 μg·mL^{-1}) for 24 h. (D) Protein levels of MTTP in H4IIE cells treated with control buffer or recombinant CTRP12 (10 μg·mL^{-1}) for

24 h. (E) Relative mRNA expression of *Hnf-4α* in H4IIE cells (n = 4–5) treated 24 h with control buffer or recombinant CTRP12 (10 μg·mL^{-1}). (F) Protein expression of HNF-4α, quantified relative to Hsc70, in H4IIE cells treated 24 h with control buffer or recombinant CTRP12 (10 μg·mL^{-1}). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Two-tailed Student's t-tests were performed for all panels

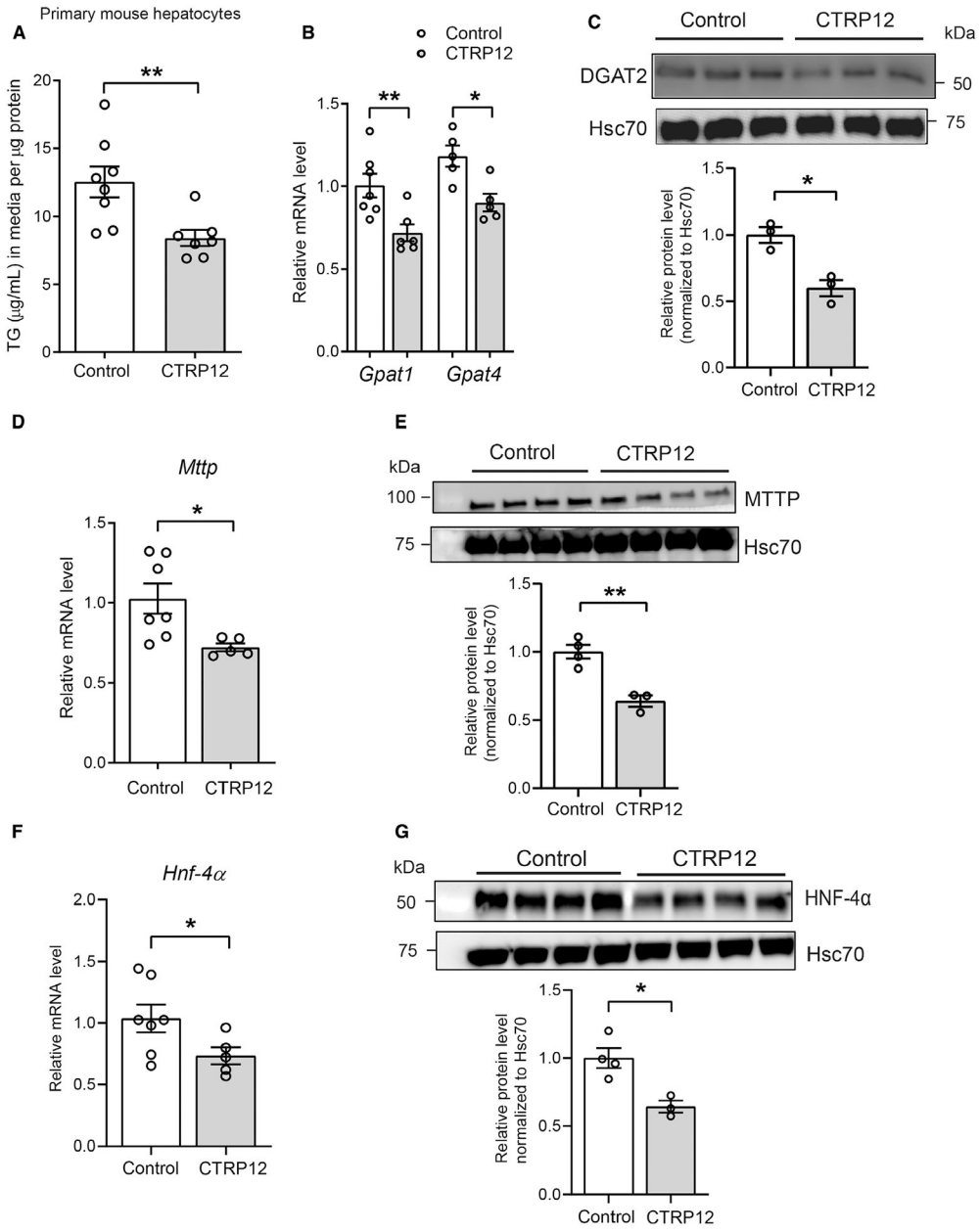

Fig. 3.

CTRP12 suppresses triglyceride synthesis and export in primary mouse hepatocytes. (A) Triglycerides secreted into the culture medium from primary mouse hepatocytes (n = 7–8) treated overnight with control buffer or recombinant CTRP12 (10 μg·mL^{-1}). (B) Relative mRNA expression of *Gpat1* and *Gpat4* in primary mouse hepatocytes (n = 5–7 per group) treated overnight with control buffer or recombinant CTRP12 (10 μg·mL^{-1}). (C) Protein expression level of DGAT2 in primary mouse hepatocytes (n = 6–7 per group) treated overnight with control buffer or recombinant CTRP12 (10 μg·mL^{-1}). (D) Relative *Mttp* mRNA and (E) MTTP protein levels (n = 7 per group) in primary hepatocytes treated overnight with control buffer or recombinant CTRP12 (10 μg·mL^{-1}). (F) Relative *Hnf-4α* mRNA and (G) HNF-4α protein levels (n = 5–7 per group) in primary hepatocytes treated

overnight with control buffer or recombinant CTRP12 (10 μg·mL^{-1}). *, $P < 0.05$; **, $P < 0.01$. Two-tailed Student's *t*-tests were performed for all panels.

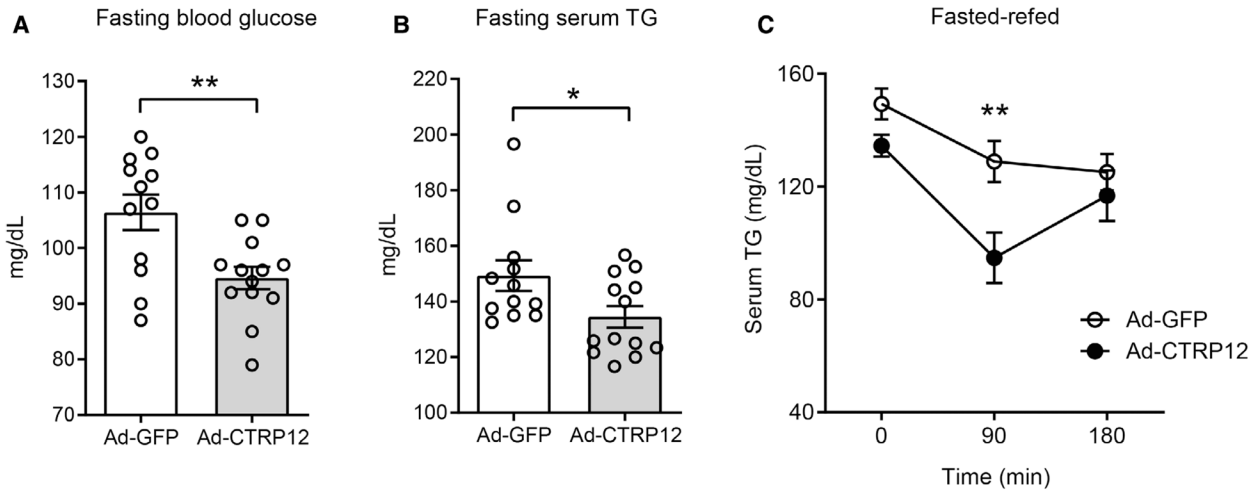

Fig. 4.

CTRP12 suppresses serum triglyceride levels *in vivo*. (A and B) Fasting levels of serum glucose (A) and triglyceride (B) in Ad-CTRP12 and Ad-GFP mice on day 8 postadenovirus injection (n = 12–13 mice per group). (C) Time course of circulating triglycerides in Ad-CTRP12 and Ad-GFP mice at 0, 90, and 180 min of postrefeeding (n = 12–13 mice per group). *, $P < 0.05$; **, $P < 0.01$. Two-tailed Student's t-tests were performed for panels A and B. For panel C, we performed 2-way ANOVA with Bonferroni post-tests.