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C1q/TNF-related Protein-12 (CTRP12), a Novel Adipokine That Improves Insulin Sensitivity and Glycemic Control in Mouse Models of Obesity and Diabetes*5

Received for publication, September 12, 2011, and in revised form, January 20, 2012 Published, JBC Papers in Press, January 24, 2012, DOI 10.1074/jbc.M111.303651

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Background: Adipose tissue-derived adipokines play important roles in regulating insulin sensitivity.

Results: CTRP12 is a hormone down-regulated in the obese state and up-regulated by an insulin-sensitizing drug. CTRP12 improves insulin sensitivity and glycemic control in mice via multiple mechanisms.

Conclusion: CTRP12 is a novel anti-diabetic adipokine.

Significance: CTRP12 is a new component of the metabolic circuitry that links adipose tissue to systemic glucose homeostasis.

Despite the prevalence of insulin resistance and type 2 diabetes mellitus, their underlying mechanisms remain incompletely understood. Many secreted endocrine factors and the intertissue cross-talk they mediate are known to be dysregulated in type 2 diabetes mellitus. Here, we describe CTRP12, a novel adipokine with anti-diabetic actions. The mRNA and circulating levels of CTRP12 were decreased in a mouse model of obesity, but its expression in adipocytes was increased by the anti-diabetic drug rosiglitazone. A modest rise in circulating levels of CTRP12 by recombinant protein administration was sufficient to lower blood glucose in wild-type, leptin-deficient ob/ob, and diet-induced obese mice. A short term elevation of serum CTRP12 by adenosine-mediated expression improved glucose tolerance and insulin sensitivity, normalized hyperglycemia and hyperinsulinemia, and lowered postprandial insulin resistance in obese and diabetic mice. CTRP12 improves insulin sensitivity in part by enhancing insulin signaling in the liver and adipose tissue. Further, CTRP12 also acts in an insulin-independent manner; in cultured hepatocytes and adipocytes, CTRP12 directly activated the PI3K-Akt signaling pathway to suppress gluconeogenesis and promote glucose uptake, respectively. Collectively, these data establish CTRP12 as a novel metabolic regulator linking adipose tissue to whole body glucose homeostasis through insulin-dependent and independent mechanisms.

Type 2 diabetes mellitus (T2DM)5 and associated pathologies are a major public health concern in the United States and worldwide (1). Efforts to treat T2DM are hampered by our incomplete understanding of the complex metabolic pathways and intertissue cross-talk involved in the control of whole body glucose and fatty acid metabolism. Many secreted endocrine factors/hormones play key roles in mediating intertissue cross-talk to maintain energy balance. In recent years, adipose tissue-secreted factors (collectively termed adipokines) have emerged as important “metabolic regulators” that act on multiple tissue types to modulate processes including food intake, insulin sensitivity, glucose and fatty acid utilization, and inflammation (2, 3). The functions of these adipokines are important for normal metabolic homeostasis; dysregulation of the pathways they modulate contributes to metabolic diseases, such as obesity, inflammation, and T2DM (2, 3).

To identify novel factors regulating intertissue cross-talk, we recently cloned a highly conserved family of secreted proteins, designated CTRP1–10 and CTRP13 based on their homology to the adipocyte-specific protein, adiponectin (4–8). All CTRPs are secreted multimeric proteins, produced by diverse tissues, and the majority is found circulating in plasma, with levels varying depending on metabolic state and genetic background of the animals (5–8). The defining feature of CTRPs is the presence of the signature “C1q/TNF-like” globular domain located at the C terminus, which is homologous to the immune complement C1q and structurally resembles that of TNF-α (9). Functional studies thus far have indicated significant roles for CTRPs in the endocrine (4–8, 10, 11), immune (12), vascular (13, 14), skeletal (15), and sensory systems (16–18).

In the present study, we characterized the function and mechanisms of action of CTRP12, a novel and distantly related proteins...
Anti-diabetic Actions of CTPR12

member of the CTRP family. Using recombinant protein administration and adenvirus-mediated expression, we provide evidence that CTPR12 is an adipokine with anti-diabetic actions in both genetic (leptin-deficient) and diet-induced mouse models of obesity and diabetes. CTPR12 ameliorates insulin resistance in part by enhancing insulin signaling in adipose tissue and liver. Based on in vivo anti-diabetic actions, decreased circulating levels in obesity, and increased expression in adipocytes upon administration of the anti-diabetic drug rosiglitazone, CTRP12 may be a potential therapeutic agent in the treatment of T2DM.

MATERIALS AND METHODS

Mice—Male wild-type, ob/ob, and diet-induced obese (DIO) mice (all on C57BL/6 genetic background) were purchased from the Jackson Laboratory. Other male mice (BALB/c, 129/Sv, FVB, DBA, and AKR) were also purchased from the Jackson Laboratory. The mice were maintained on a standard chow diet, 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 Johns Hopkins University School of Medicine.

Antibodies and Chemicals—AMPK activator (aminomimidazole carboxamide ribonucleotide) (AICAR) (19) was obtained from Calbiochem; P3K inhibitor (LY-294002) was from Cell Signaling Technology. Phospho-IRS1 (Tyr-612), phospho-p44/42 MAPK (Erk1/2; Thr-202/Tyr-204), phospho-FoxO1 (Ser-256), phospho-AKT (Thr-308), phospho-p44/42 MAPK (Erk1/2; Thr-202/Tyr-204), phospho-FoxO1 (Ser-256), and phospho-PI3K (80) were obtained from Cell Signaling Technology. Phospho-IRS1 (Tyr-612) was obtained from BIOSOURCE. Anti-phosphotyrosine antibody (clone 4G10) was obtained from Sigma. Antibodies that recognize phospho-AKT (Thr-308), phospho-p44/42 MAPK (Erk1/2; Thr-202/Tyr-204), phospho-FoxO1 (Ser-256), IRS-1, Akt, and p44/42 MAPK were obtained from Cell Signaling Technology. Phospho-IRS1 (Tyr-612) was obtained from BIOSOURCE. Anti-phosphotyrosine antibody (clone 4G10) was obtained from Millipore. Anti-Insulin receptor antibody was obtained from Cell Signaling Technology. Rabbit anti-CTPR12 polyclonal antibody was produced as previously described (6). Anti-CTPR12 antibody specifically recognizes CTPR12, not other CTRPs (supplemental Fig. S9).

Cloning—CTPR12 (also called C1qTNF12) was cloned based on three overlapping expressed sequence tag sequences, BY704358, B1693294, and COO44841, from a mouse testis cDNA pool (Clontech). Primers were designed to amplify the entire coding region of mouse CTPR12 from a mouse testis cDNA pool (Clontech). The forward and reverse primers used in the 33-cycle PCR reactions were 5’-CGGAGACTGAGCC-ATGTTGGGCTGG-3’ and 5’-CGTTCCTTACAGCTCCGCT-AGGTACC-3’, respectively. The PCR product was purified and cloned into pCRII TOPO vector (Invitrogen), and the entire cDNA insert was sequenced. The sequence data for CTPR12 (C1qTNF12) was deposited into the NCBI GenBank database and was assigned the accession number DQ002404.

Quantitative Real Time PCR Analysis—CTPR12 tissue expression analysis was performed using mouse and human tissue cDNA panels (Clontech). Every mouse tissue was pooled from 200–1000 mice, and every human tissue was pooled from 3–15 individuals. Thus, the expression of CTPR12 in each tissue represents an average value from the pooled cDNA. Other RNAs were isolated from tissues or cell lines using TRIzol® and reverse transcribed using Superscript II RNase H-reverse transcriptase (Invitrogen). The quantitative RT-PCR analyses were performed on an Applied Biosystems Prism 7500 sequence detection system. The samples were analyzed in 25-µl reactions according to the standard protocol provided in the SyBr® Green PCR Master Mix (Applied Biosystems). Expression of gluconeogenic enzyme genes (G6Pase and PEPC) in H411E cells was analyzed after cells had been treated with vehicle or CTPR12 (10 µg/ml) for 24 h. All of the expression levels were normalized to corresponding 18 S RNA levels. Primers used in real time PCR included the following: CTPR12, forward 5’-CGATTTCACAGCCCCAGTCTC-3’ and reverse 5’-GTGCAAGCTGCCGCAAAAGC-3’; G6Pase, forward 5’-CGACTGCATATCTCCAAGTG-3’ and reverse 5’-GGTGAAACGAGTCTCAGCAAC-3’; PEPC, forward 5’-CTGCAATAACGGAAGTCTGGACTTACC-3’ and reverse 5’-GAAGGCCCTGTTCTGACAC-3’; 18S, forward 5’-ATGCTGGGACAGTGACCTGG-3’ and reverse 5’-CCTTGAATGTTGTCAATGAG-3’; MCP-1, forward 5’-TTAAAACCTGGATGGAACCAA-3’ and reverse 5’-GCAATCTGCTACCACTGCTG-3’ and reverse 5’-GGAGACATCCGCTGTAG-3’ and reverse 5’-GCAATTTATCCCATGAAACAGC-3’ and reverse 5’-GGCCTCACAACATCCGAA-3’.

Protein Purification—Recombinant CTPR12 was produced and purified as described (6). Briefly, GripTite™ HEK 293 cells were transfected with pCDNA3.1, encoding a C-terminal FLAG-tagged CTPR12, using Lipofectamine 2000 (Invitrogen). Supernatants (serum-free Opti-MEM) of cells were collected and subjected to affinity chromatography using ANTI-FLAG® M2 affinity gel (Sigma) according to the manufacturer’s protocol. Purified protein was dialyzed against 25 mM HEPES (pH 8.0) containing 135 mM NaCl, concentrated with a 10-kDa cut-off Amicon Ultra centrifugal filter (Millipore). Protein concentration was determined using the Coomassie Plus Protein Assay (Thermo Scientific). Purity of the protein (>95%) was determined by SDS-PAGE analysis and Coomassie Blue staining.

N-terminal Sequencing of gCTRP12—Recombinant CTPR12 was electrophoresed onto PVDF membrane. The band corresponding to gCTRP12 was excised. Sequencing was performed at the mass spectrometry facility at Johns Hopkins University School of Medicine.

Glucose Uptake—3T3-L1 cells were cultured and differentiated as described (7). Glucose uptake in 3T3-L1 adipocytes was performed as described (20). In brief, 3T3-L1 adipocytes were serum-starved in low glucose DMEM for 2 h. 3T3-L1 adipocytes were treated with vehicle or CTPR12 (10 µg/ml) for 30 min, and/or with insulin (10 nM) for 15 min, and/or with 25 mM HEPES-NaOH, pH 7.4, 120 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 1.3 mM CaCl2, 1.3 mM KH2PO4, and 0.5% BSA for 10 min. Uptake was stopped by aspirating the media and extensive washing with ice-cold PBS buffer. The cells were lysed with 0.1% Triton in 120 mM NaCl, 5 mM KCl, 1.2 mM MgSO4, 1.3 mM CaCl2, 1.3 mM KH2PO4, and 0.5% BSA for 10 min. Uptake was stopped by aspirating the media and extensive washing with ice-cold PBS buffer. The cells were lysed with 0.1% Triton in...
PBS buffer. An aliquot of cell lysate from each sample was obtained for protein content analysis using BCA assay kit (Pierce). Radioactivity of cell lysates was counted in Ecoscint® scintillation mixture (National Diagnostics) using a Beckman LS-6000 liquid scintillation counter. Radioactivity in each sample was normalized against protein content.

GLUCOSE PRODUCTION AND MEASUREMENT—This assay was performed as described [21]. In brief, H4IIE hepatoma cells were cultured to near confluence in low glucose DMEM containing 10% FBS and antibiotics. The cells were incubated for overnight in glucose production buffer (glucose-free DMEM, pH 7.4, supplemented with 20 mM sodium lactate and 2 mM sodium pyruvate without phenol red). Then cells were treated with vehicle buffer or CTRP12 (5 µg/ml). An aliquot of the medium was taken from each sample for the measurement of glucose concentration using the Amplex Red® glucose assay kit (Invitrogen). Glucose concentration was corrected for cell number variations based on protein content in each sample as measured by the BCA assay. For quantitative RT-PCR analysis of gluconeogenic genes (G6Pase and PEPCK), H4IIE hepatoma cells were cultured to near confluence in low glucose DMEM containing 10% FBS and antibiotics. The cells were then treated with vehicle buffer or CTRP12 (10 µg/ml) for 24 h. RNAs were isolated from the cells and reverse-transcribed into cDNA. Quantitative RT-PCR analyses were performed as described above.

Injection of Recombinant CTRP12 into Mice—C57BL/6 male mice were purchased from the Jackson Laboratory at 7 weeks of age. They were allowed to acclimate to the animal facility at Johns Hopkins University for 2 weeks. These mice were 9 weeks of age. They were allowed to acclimate to the animal facility at Johns Hopkins University for 2 weeks. These mice were 9 weeks of age at the time of protein injection. On the morning of injection, food was removed from the cage 3 h before recombinant protein administration and during the course of the experiment. Drinking water was provided to mice throughout the experiment. Either vehicle control or CTRP12 was injected into mice intraperitoneally; CTRP12 was injected at a dose of 3.5 µg/g of body weight. Glucose concentration from tail vein blood was measured at indicated time intervals using a glucometer (BD Pharmingen). Serum was collected at indicated time intervals, and the serum concentrations of insulin and glucagon were measured using kits from Millipore.

Insulin Secretion Assay—This assay was performed essentially as described [22], with the following changes: INS-1 cells were seeded at 0.1 million cells/well in 96-well plates and cultured until 100% confluence when the assay was performed. The insulin concentration was determined by the insulin ELISA kit (Millipore) and normalized against the protein concentration of the cell lysate in each well.

Adenovirus Preparation and Infection—Adenovirus encoding GFP or CTRP12 was prepared using the AdEasy™ adeno-viral vector system (Stratagene) according to the manufacturer’s protocol. In brief, C-terminal FLAG-tagged CTRP12 cDNA was cloned into the pShuttle-IRES-hrGFP-2 vector (shuttle vector). The empty shuttle vector was used to generate the GFP virus. The shuttle vector was linearized and recombined with pAdEasy-1 plasmid in BJ5183 Escherichia coli. Recombinants were identified and produced in bulk to generate the recombinant adenovirus plasmid DNA. Purified recombinant adenovirus plasmid DNA was digested with Pacl and used to transfect HCK 293 cells. Adenovirus was amplified in HEK 293 cells, purified using CsCl density centrifugation, and then dialyzed in TD buffer (137 mM NaCl, 6 mM KCl, 0.7 mM Na2HPO4, 25 mM Tris-HCl, pH 8.0). GFP- or CTRP12-encoding adenovirus was injected via tail veins into C56BL/6 male mice at a dose of 5 × 1012 viral particles/kg of body weight.

Gluconeogenic Genes (G6Pase and PEPCK), H4IIE hepatoma cells were cultured to near confluence in low glucose DMEM containing 10% FBS and antibiotics. The cells were then treated with vehicle buffer or CTRP12 (10 µg/ml) for 24 h. RNAs were isolated from the cells and reverse-transcribed into cDNA. Quantitative RT-PCR analyses were performed as described above.

GTT was performed on day 5 post-adenovirus infection, when the expression of CTRP12 was at its peak. GFP- and CTRP12-expressing mice were fasted for 7 h before the GTT. On the day of experiment, glucose was intraperitoneally injected into mice at a dose of 1 mg/kg of body weight. Blood glucose was measured at the indicated time points using a glucometer (BD Pharmingen). Serum insulin and glucagon were measured using ELISA kit from Millipore.

Insulin Tolerance Test (ITT)—ITT was performed on day 4 post-adenovirus infection. Food was removed 2 h prior to insulin injection. Insulin was intraperitoneally injected into mice at a dose of 1 unit/kg of body weight. Blood glucose was measured at indicated time points using a glucometer (BD Pharmingen).

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Meal Tolerance Test—GFP- and CTRP12-expressing mice were fasted overnight. Blood glucose levels were measured, and sera were obtained before mice were given unlimited access to food pellets for 1 h. Blood glucose and sera were obtained again after 1 h of refeeding. Food consumption was measured during this 1-h refeeding.

Blood Chemistry Analysis—Fasting metabolic parameters were obtained after a 7-h fast. Fed parameters were obtained in ad libitum fed mice with 2-h food removal. Peptide and protein hormones were measured using corresponding ELISA or luminex kits from Millipore. Serum activities of alanine transaminase and aspartate aminotransferase were measured at the Mouse Phenotyping and Pathology Core at Johns Hopkins University School of Medicine.

Statistics—All of the statistical analyses were performed using two-tailed Student’s t test in Excel. p < 0.05 was considered significant. All of the quantification results with error bars are expressed as the means ± S.E.

RESULTS

Identification of CTRP12 as an Adipokine—CTRP12 was identified in silico as possessing a globular C1q/TNF-like domain, the signature domain of CTRPs. Limited sequence similarity to other CTRPs, including adiponectin (21% identity) and C1q (~20% identity), make it a distantly related member. The protein is evolutionarily conserved (supplemental Fig. S1 and Table S1) and has four basic domains (Fig. 1A). CTRP12 has a protease cleavage motif, 90KKSR93, with the cleavage site mapped to Lys-91 by N-terminal sequencing (Fig. 1A, lane 1 and Table S1). In circulating mouse and human sera, gCTRP12 is the predominant form (Fig. 1, lane 2). CTRP12 was identified and produced in bulk to generate the recombinant adenovirus plasmid DNA. Purified recombinant adenovirus plasmid DNA was digested with Pacl and used to transfect HCK 293 cells. Adenovirus was amplified in HEK 293 cells, purified using CsCl density centrifugation, and then dialyzed in TD buffer (137 mM NaCl, 6 mM KCl, 0.7 mM Na2HPO4, 25 mM Tris-HCl, pH 8.0). GFP- or CTRP12-encoding adenovirus was injected via tail veins into C56BL/6 male mice at a dose of 5 × 1012 viral particles/kg of body weight.

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In mice, CTRP12 is more widely expressed (Fig. 1D). In humans, it is expressed predominantly by adipose tissue (Fig. 1E); mouse white and brown adipose tissues express comparable amounts of CTRP12 (data not shown). The higher expression of human CTRP12 mRNA in adipose tissue correlates with its higher serum levels (Fig. 1B, lane 3). CTRP12 is highly induced during adipogenesis (Fig. 1F). Additionally, insulin increases both transcript (Fig. 1G) and protein levels of CTRP12 in adipocytes (Fig. 1H and I). Interestingly, administration of rosiglitazone, a pro-adipogenic and an insulin-sensitizing drug (23, 24), also increases the expression of CTRP12 mRNA in adipocytes (Fig. 1J). In contrast, both the mRNA and serum levels of CTRP12 are suppressed in insulin-resistant obese (ob/ob) mice (Fig. 1, K and L). These results indicate that CTRP12 expression is positively regulated by insulin and rosiglitazone and is dysregulated under the condition of obesity.

Recombinant CTRP12 Administration Decreases Blood Glucose in WT, ob/ob, and DIO Mice—To determine the metabolic functions regulated by CTRP12, we administered recombinant CTRP12 to wild-type C57BL/6 male mice and examined changes in glucose dynamics. Secreted recombinant CTRP12 containing both fCTRP12 and gCTRP12 was affinity-purified from the supernatant of transfected HEK 293 cells (see “Materials and Methods”).

In mice, CTRP12 is a novel insulin-regulated adipokine down-regulated in obesity and up-regulated by an anti-diabetic drug.
Intraperitoneal injection of CTRP12 at a dose of 3.5 μg/g of body weight into WT mice resulted in an acute 40% increase in serum CTRP12 over the physiological level (Fig. 2A). Concomitantly, there was a significant lowering in blood glucose with a maximum of 50 mg/dl reduction in CTRP12-treated WT mice compared with vehicle-injected controls (Fig. 2B). Because food was removed 2–3 h prior to recombinant protein administration and during the course of the experiment, we expected that blood glucose in vehicle-injected mice would also decrease over time. Unexpectedly, we observed a maximum 70% increase in serum insulin levels after CTRP12 administration (Fig. 2C). Consistent with this in vivo observation, in rat pancreatic β-cells (INS-1), CTRP12 potentiated glucose-stimulated insulin secretion at low, intermediate, and high glucose concentrations (Fig. 2D). A concentration of 5 μg/ml of CTRP12 was sufficient to induce maximum insulin secretion from INS-1 cells (Fig. 2E). The observed increase in serum insulin concentration after CTRP12 administration to WT mice is unlikely to be caused by a decrease in insulin clearance; in vitro, recombinant CTRP12 treatment did not change the rate of insulin clearance over time in H4IIE hepatocytes (Fig. 2F).

We next tested whether CTRP12 can lower blood glucose in mouse models of diabetes. We used two diabetic mouse models: leptin-deficient ob/ob mice and DIO mice, both of which are on the C57BL/6 genetic background. In ob/ob mice, intraperitoneal administration of recombinant CTRP12 at a dose of 1.5 μg/g of body weight caused a 20% increase in serum CTRP12 over baseline levels (Fig. 2G). This change was accompanied by a 50 mg/dl decrease in blood glucose concentration over a 2-h period (Fig. 2H).
unchanged in ob/ob mice injected with CTRP12 (Fig. 2I) compared with vehicle-injected mice. Given that ob/ob mice are already hyperinsulinemic, it is not surprising that CTRP12 failed to induce further increases in serum insulin concentration as observed in WT mice.

Administration of CTRP12 (1.5 μg/g of body weight) to DIO mice fed a high fat diet for 14 weeks caused a modest increase in the serum level of CTRP12 (Fig. 2J). However, the glucose-lowering effect of CTRP12 was much more potent in DIO mice, with a maximum ~100 mg/dl decrease in blood glucose concentration in the CTRP12-treated group (Fig. 2K). At 3 h and beyond, DIO mice injected with CTRP12 had blood glucose levels of ~50 mg/dl (Fig. 2H), well below the normal fasting blood glucose concentration in mice. As with ob/ob mice, DIO mice injected with recombinant CTRP12 showed no changes in insulin levels (Fig. 2L). Together, these results indicate that recombinant CTRP12 administration at physiologic doses lowers blood glucose levels in WT and obese mice, with the greatest effect observed in DIO mice.

CTRP12 Enhances Insulin Sensitivity in WT, ob/ob, and DIO Mice—To extend our study on the acute metabolic effects of recombinant protein administration, we next sought to address the metabolic consequences of short-term ectopic expression of CTRP12 in mice using adenoviral vectors. Adenovirally mediated overexpression of CTRP12 did not result in body weight changes compared with GFP-expressing mice over the course of the experiment (supplemental Fig. S2). In WT mice, ectopic expression of CTRP12 caused a maximum 3-fold elevation in serum CTRP12 levels (Fig. 3A). Blood glucose levels were 20 mg/dl lower in the fed state in CTRP12-expressing mice (Fig. 3B) but were not significantly different in the fasted state. Serum insulin levels in these mice were ~45% higher in the fed state but were not different in the fasted state (Fig. 3C). The fasted and fed glucagon levels were not different between the two groups (data not shown).

Assessment of whole body insulin sensitivity using the homeostatic model assessment insulin resistance (HOMA-IR) index (25) revealed a trend toward a decreased insulin resistance index in CTRP12-expressing WT mice (Fig. 3D). Consistent with this, WT mice expressing CTRP12 showed a much faster rate of glucose disposal over time in a GTT (Fig. 3E), despite no changes in insulin or glucagon levels during the GTT (Figs. 3F and data not shown). These results indicate that WT mice expressing CTRP12 are more insulin-sensitive.

In ob/ob mice, expression of CTRP12 resulted in a 2–3-fold increase of serum CTRP12 levels over GFP-expressing mice (Fig. 3G). Fed and fasted glucose and insulin levels in ob/ob mice expressing CTRP12 were much lower compared with mice expressing control GFP (Fig. 3, H and I). The significant reduction in fasted blood glucose and insulin levels in ob/ob mice indicated decreased insulin resistance, as reflected by the HOMA-IR index (Fig. 3J). Consistent with improved insulin sensitivity, ob/ob mice expressing CTRP12 had a much faster rate of glucose disposal in a GTT (Fig. 3K) with significantly lower insulin levels over the course of the experiment (Fig. 3L). These results indicate that CTRP12-expressing ob/ob mice are more insulin-sensitive. In an ITT, ob/ob mice expressing CTRP12 had overall lower blood glucose levels, as reflected by a smaller area under the curve (AUC) (Fig. 3M). This was largely due to lower glucose concentrations at the beginning of the experiment.

In DIO mice, the adenoviral vector effected a 2–3-fold increase in serum CTRP12 levels over GFP-expressing mice (Fig. 3N). DIO mice expressing CTRP12 had lower fasted blood glucose levels (Fig. 3O) and significantly lower fed and fasted insulin levels (Fig. 3P). The reduction in fasted blood glucose and insulin levels in DIO mice expressing CTRP12 resulted in greatly reduced HOMA-IR index (Fig. 3Q). Consistent with improved insulin sensitivity, DIO mice expressing CTRP12 showed a greater rate of glucose disposal with considerably lower insulin levels in a GTT (Figs. 3, R and S). This reflects greater insulin sensitivity in DIO mice expressing CTRP12. Although DIO mice expressing CTRP12 had lower blood glucose levels in an ITT (Fig. 3T), it was largely due to lower starting concentrations of blood glucose. Combined, these data demonstrate that a short-term elevation of circulating CTRP12 levels by adenoviral method can significantly improve whole body insulin sensitivity and glucose homeostasis in WT as well as obese (ob/ob and DIO) mice.

CTRP12 Reduces Postprandial Insulin Resistance in DIO Mice—Because CTRP12 is up-regulated by insulin (Fig. 1, H and I) and insulin is a major hormone regulating metabolism in the postprandial state, we hypothesized that CTRP12 may play a role in regulating postprandial insulin sensitivity as well. To examine this possibility, we performed a meal tolerance test in DIO mice expressing control GFP or CTRP12. Mice were fasted overnight and then given unlimited access to food for 1 h in the refeeding phase. No difference was observed in food intake in the refeding period between GFP- and CTRP12-expressing DIO mice (Fig. 4A). Despite similar postprandial blood glucose levels (Fig. 4B), DIO mice expressing CTRP12 had much lower insulin levels in the refed state (Fig. 4C), indicating a decrease in postprandial insulin resistance in these mice. A notable reduction (~50%) in glucose-dependent insulinotropic polypeptide (GIP) levels was also observed in DIO mice expressing CTRP12 (Fig. 4D). Because GIP promotes postprandial insulin secretion from pancreatic β cells, a decrease in GIP levels may have contributed to the marked reduction in insulin levels seen in the refed state in CTRP12-expressing DIO mice (Fig. 4D). In our experiment, we failed to detect glucagon-like peptide (GLP-1) using the milliplex assay platform, and consequently, it remains to be determined whether postprandial levels of GLP-1 in serum also change in mice expressing CTRP12 compared with GFP controls. The meal tolerance test results independently confirmed the insulin-sensitizing function of CTRP12 in DIO mice in the context of normal physiology associated with meal consumption.

CTRP12 Does Not Modulate Serum Fatty Acid Levels, Adipose Tissue Inflammation, or Adipocyte Size, but Reduces Resistance Expression—In the diabetic state, serum nonesterified fatty acid levels are often elevated, and this may indirectly contribute to hyperglycemia (26). Thus, we determined serum nonesterified fatty acid levels in CTRP12-expressing mice. No difference in nonesterified fatty acid levels was observed in WT, DIO, and ob/ob mice expressing GFP or CTRP12 (supplemental Fig. S4A). Chronic low grade inflammation is tightly associated with and may underlie the insulin-resistant state (27). Thus, to test...
FIGURE 3. CTRP12 improves insulin sensitivity in WT, ob/ob, and DIO mice. A, immunoblot quantification of serum CTRP12 levels following tail vein injection of adenovirus into WT C57BL/6 mice (male, 9 weeks old, n = 6). B and C, fed and fasted blood glucose (B) and serum insulin (C) levels of GFP- or CTRP12-expressing WT mice (n = 8–9). D, HOMA-IR index of WT mice expressing GFP or CTRP12. E, GTT of WT mice expressing GFP (n = 8) or CTRP12 (n = 9). F, serum insulin levels during the course of GTT (n = 8–9). G, immunoblot quantification of serum CTRP12 levels following adenovirus injection into ob/ob mice (male, 10 weeks old, n = 6). H and I, fed and fasted glucose (H) and insulin (I) levels of GFP- or CTRP12-expressing ob/ob mice (n = 6). J, HOMA-IR index of ob/ob mice expressing GFP or CTRP12 (n = 6). K, GTT of ob/ob mice expressing GFP or CTRP12 (n = 6). L, serum insulin levels during the course of GTT in ob/ob mice expressing GFP or CTRP12 (n = 6). M, blood glucose levels and area under the curve (AUC) of glucose in the ITT in ob/ob mice expressing GFP or CTRP12 (n = 6). N, immunoblot quantification of serum CTRP12 levels following adenovirus injection into DIO mice (male, 20 weeks old, n = 6). O and P, fed and fasted glucose (O) and insulin (P) levels of GFP- or CTRP12-expressing DIO mice (n = 6). Q, HOMA-IR index of DIO mice expressing GFP or CTRP12 (n = 6). R, GTT of DIO mice expressing GFP or CTRP12 (n = 6). S, serum insulin levels during the course of GTT in DIO mice expressing GFP or CTRP12 (n = 6). T, blood glucose levels and area under the curve (AUC) in the ITT in DIO mice expressing GFP or CTRP12 (n = 6). All of the data are expressed as the means ± S.E. #, p < 0.001; **, p < 0.01; *, p < 0.05.
whether CTRP12 reduced insulin resistance through potential anti-inflammatory actions, we measured serum levels of two key inflammatory cytokines, TNF-α and IL-6, in mice expressing control GFP or CTRP12. As shown in supplemental Fig. S3, overexpressing CTRP12 did not lower circulating levels of TNF-α and IL-6 in WT, ob/ob, or DIO mice. Further, expression of inflammatory genes in adipose tissue, specifically IL-6, MCP-1, TNF-α, and MIP-1α, did not differ between GFP- and CTRP12-expressing ob/ob and DIO mice (supplemental Fig. S4, B and C). In contrast to TNF-α and IL-6, serum levels of resistin, an adipokine known to induce insulin resistance in mice (28), were significantly reduced in ob/ob and DIO mice expressing CTRP12 (Fig. 5, B and C). However, unlike in ob/ob and DIO mice, overexpressing CTRP12 in WT mice did not alter resistin levels (Fig. 5A). The lowering of resistin levels in obese mice suggests an altered adipokine-mediated tissue cross-talk in the obese state. The lowering of resistin levels is specific because CTRP12 overexpression did not alter circulating levels of other adipokines, such as adiponectin, leptin, and plasminogen activator inhibitor-1 (Fig. 5). Consistent with the in vivo data, recombinant CTRP12 directly lowered resistin secretion from cultured 3T3-L1 adipocytes in vitro (Fig. 5D), whereas it had no effect on leptin or adiponectin secretion (Fig. 5, G and K). Histological examination of adipose tissue derived from epididymal fat pads of CTRP12-expressing DIO mice did not reveal any alteration in adipocyte number or size when compared with fat pads from GFP-expressing mice (supplemental Fig. S4, D and F). Together, these data indicate that CTRP12 reduces resistin levels in the obese state, and the CTRP12-mediated improvements in glucose tolerance and insulin sensitivity in ob/ob and DIO mice can be uncoupled from its effect on the inflammatory state or cell size of adipose tissue.

**CTRP12 Enhances Insulin Signaling in Adipose Tissue and Liver**—To test whether the improved metabolic profile of CTRP12-expressing mice is correlated with enhanced insulin signaling, we analyzed the phosphorylation state of major insulin signaling molecules in three major metabolic tissues: liver, adipose tissue, and skeletal muscle. Examination of insulin signaling using Western blot showed an increase in tyrosine phos-
Phosphorylation of insulin receptor substrate (IRS-1) in adipose tissue of CTRP12-expressing WT mice relative to GFP-expressing controls (Fig. 6A). Consistent with this, protein kinase B/Akt, a signaling molecule downstream of IRS-1, also showed enhanced phosphorylation in adipose tissue of CTRP12-expressing WT mice (Fig. 6A). In contrast, p44/42 MAPK, a signaling molecule that can also be activated by insulin, did not show any changes in its phosphorylation state (Fig. 6A). In liver from CTRP12-expressing WT mice, we observed similar increases in the phosphorylation of IRS-1 and Akt (Fig. 6B). In ob/ob mice, which are both obese and insulin-resistant, overexpressing CTRP12 also significantly improved insulin signaling, reflected by enhanced IRS-1 and Akt phosphorylation in adipose tissue and liver (Fig. 6, C and D). Consistent with improved insulin signaling in the liver of ob/ob mice, mRNA expression of G6Pase and PEPCK, two gluconeogenic enzyme genes nor-

**FIGURE 6. CTRP12 enhances insulin signaling in adipose tissue and liver of normal and diabetic mice.** A and B, representative immunoblot and quantification of phosphorylation of IRS-1 (Tyr-612), Akt (Thr-308), and p44/42 MAPK (Tyr-202/Tyr-204) in adipose tissue (A) and liver (B) of WT mice expressing GFP or CTRP12 (n = 6). C and D, representative immunoblot and quantification of phosphorylation of IRS-1 (Tyr-612), Akt (Thr-308), and p44/42 MAPK (Thr-202/Tyr-204) in adipose tissue (C) and liver (D) of ob/ob mice expressing GFP or CTRP12 (n = 6). E, real time PCR analysis of G6Pase and PEPCK expression in the liver of ob/ob mice expressing GFP or CTRP12 (n = 6). F, real time PCR analysis of G6Pase and PEPCK expression in H4IIE hepatocytes treated with vehicle control or CTRP12 (10 μg/ml) (n = 6). Expression values were normalized to β-actin or 18 S rRNA transcript levels.
mally suppressed by insulin, were also strikingly reduced in the liver of ob/ob mice (Fig. 6E). The direct effect of CTRP12 in suppressing hepatic gluconeogenic gene expression was further confirmed in vitro; recombinant CTRP12 potently decreased G6Pase and PEPCK mRNA expression in rat H4IIE hepatocytes (Fig. 6F). Unlike in liver and adipose tissue, overexpressing CTRP12 did not alter the phosphorylation state of MAPK or Akt in skeletal muscles of WT or ob/ob mice (supplemental Fig. S5), indicating that CTRP12 preferentially acts on adipose tissue and liver in vivo.

To determine whether adenovirus infection alters hepatic physiology and function, we examined the expression of inflammatory genes (IL-1β, IL-6, and TNF-α) and serum activities of alanine transaminase and aspartate aminotransferase, two common serum markers of liver damage. Although CTRP12-expressing ob/ob and DIO mice had lower levels of aspartate aminotransferase and alanine transaminase serum activities (supplemental Fig. S6A), no differences were observed in the hepatic expression of inflammatory genes between WT, ob/ob, and DIO mice expressing GFP or CTRP12 (supplemental Fig. S6B).

CTRP12 Activates the PI3K-Akt Signaling Pathway to Suppress Gluconeogenesis in Hepatocytes and Promote Glucose Uptake in Adipocytes—To uncover signaling pathways directly activated by CTRP12 and to address whether CTRP12 acts independently of insulin, we utilized insulin-responsive cell lines to investigate the mechanisms of action of CTRP12. Although recombinant CTRP12 did not induce insulin recep-

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**FIGURE 7.** CTRP12 activates the PI3K-Akt signaling pathway to regulate hepatic glucose metabolism. A, CTRP12 (10 μg/ml) does not increase the phosphorylation of IR in H4IIE hepatocytes. B–D, time course of phosphorylation of IRS1 (Tyr-612) (B), Akt (Thr-308) (C), and p44/42 MAPK (Thr-202/Tyr-204) (D) induced by CTRP12 treatment (10 μg/ml). E, CTRP12 (10 μg/ml, 24-h incubation) decreases gluconeogenesis in rat H4IIE hepatocytes (n = 3). F, suppression of gluconeogenesis by CTRP12 (10 μg/ml, 24-h incubation) in H4IIE hepatocytes is inhibited by LY-294002 (20 μM, 24-h coincubation). G and H, the increase of Akt (Thr-308) (G) and FoxO1 (Ser-256) (H) phosphorylation induced by CTRP12 (10 μg/ml) is inhibited by LY-294002 (20 μM). I, inhibition of gluconeogenesis in H4IIE hepatocytes by CTRP12 (10 μg/ml) in the presence of various concentrations of insulin. Ins, insulin (10 nM); LY, LY-294002; 12, recombinant CTRP12; Ctrl, control. All of the data are expressed as the means ± S.E. #, p < 0.001; **, p < 0.01; *, p < 0.05. α, p < 0.05 compared with control; β, not-significant compared with control; γ, p < 0.05 compared with insulin; δ, p < 0.05 compared with CTRP12. IB, immunoblot; IP, immunoprecipitation.
tor (IR) phosphorylation in rat H4IIE hepatocytes (Fig. 7A), it robustly enhanced IRS-1 and Akt phosphorylation (Fig. 7B and C). Although phosphorylation of p44/42 MAPK was not altered in CTRP12-expressing WT and ob/ob mouse liver, recombinant CTRP12 acutely induced its phosphorylation in H4IIE hepatocytes in vitro (Fig. 7D). In the absence of insulin, recombinant CTRP12 can still suppress de novo glucose production in H4IIE hepatocytes (Fig. 7E), likely via inhibition of gluconeogenic enzyme gene expression (Fig. 6F). The observed suppression of gluconeogenesis by CTRP12 is in part dependent on pI3K-Akt signaling; the addition of a pI3K-specific inhibitor, LY29004, partially abolished the effect of CTRP12 on gluconeogenesis in vitro (Fig. 7F). Similarly, Akt activation by CTRP12 was inhibited by LY29004 in hepatocytes (Fig. 7G). FoxO1, a downstream target of Akt and an important regulator of hepatic gluconeogenesis (29), also showed increased phosphorylation in hepatocytes in response to CTRP12 treatment (Fig. 7H). This increase in phosphorylation was LY29004-sensitive, similar to insulin-induced Akt and FoxO1 phosphorylation (Fig. 7, G and H). These results indicate that CTRP12 activates Akt through pI3K downstream of IRS-1.

We then addressed whether CTRP12 synergizes with insulin at the cellular level to control hepatic gluconeogenesis. Incubation of H4IIE hepatocytes with insulin showed a 50% suppression of gluconeogenesis at the IC50 concentration of ~100 pm (Fig. 7I), as previously reported (21). In the presence of recombinant CTRP12, we observed further inhibition of gluconeogenesis, but the IC50 of insulin remained the same (Fig. 8I). This finding indicates that the suppression of gluconeogenesis by CTRP12 and insulin is additive rather than synergistic (Fig. 7). In differentiated 3T3-L1 adipocytes, acute treatment with recombinant CTRP12 strongly induced the phosphorylation of IRS-1 and Akt (Fig. 8, A and B). Similar to H4IIE hepatocytes, recombinant CTRP12 also acutely induced p44/42 MAPK phosphorylation (Fig. 8C). Treatment of 3T3-L1 adipocytes with CTRP12 led to a modest increase in glucose uptake, and this increase was nonadditive to that induced by 10 nm insulin (Fig. 8D), suggesting that CTRP12 possibly acts on the same signaling targets as insulin in adipocytes. The increases in glucose uptake and activation of Akt by CTRP12 in adipocytes were inhibited by LY29004 (Fig. 8, E and F), indicating that the pI3K-Akt signaling pathway mediates the effect of glucose uptake by CTRP12. These data demonstrate a direct role of CTRP12—indepedent of insulin—in regulating glucose metabolism in hepatocytes and adipocytes.

Consistent with the in vivo data indicating no changes in the phosphorylation states of Akt and p44/42 MAPK in skeletal muscle of mice overexpressing CTRP12 (supplemental Fig. S5), recombinant CTRP12 treatment had no effect on the phosphorylation states of these signaling molecules in rat L6 myotubes (supplemental Fig. S7).

**DISCUSSION**

CTRP12 was identified in an in silico screen for proteins possessing the C1q/TNF-like domain. With low amino acid sequence identity (less than 22%) compared with other family members, CTRP12 is distantly related to adiponectin and other CTRPs that we recently identified and characterized (4–8, 30). As part of a recent large scale cDNA sequencing project, a cDNA that encodes CTRP12 was also designated as FAM132a (family with sequence similarity 132, member A) (31), the function of which is unknown. We showed that CTRP12 is an endocrine factor secreted by adipocytes that circulates in plasma. Although CTRP12 mRNA and circulating levels are suppressed in an obese mouse model, expression in adipocytes is up-regulated by the anti-diabetic drug rosiglitazone, suggesting a possible role in promoting insulin sensitivity. Indeed, our functional studies revealed that CTRP12 is a novel adipokine that enhances systemic insulin sensitivity and lowers blood glucose in wild-type mice, as well as in various mouse models of obesity and diabetes.

The relative adipose tissue-restricted expression pattern of CTRP12 in humans, and its more widespread expression pattern in mice suggests an intrinsic difference in the regulation of CTRP12 expression between the two species. Higher expression of human CTRP12 mRNA also correlated with its higher circulating levels compared with the mouse counterpart. Thus, to ascertain the potential relevance of our study to human physiology, we queried the expression data of human CTRP12 in the Gene Expression Omnibus, a public repository of microarray data sets. In one unpublished data set submitted to the Gene Expression Omnibus (accession number GDS3688), C. M. Aguiler et al. reported global differential expression of genes in omental adipose tissue from obese prepubertal children and control lean subjects. The expression of CTRP12, designated as FAM132a in this data set, is significantly reduced in obese prepubertal children compared with control subjects (supplemental Fig. S8). This result is consistent with our data showing that the mRNA and circulating levels of CTRP12 are significantly reduced in obese (ob/ob) mice, highlighting not only amino acid sequence conservation (70% identity) between full-length human and mouse CTRP12, but also their regulation in mice and humans. As with the mouse counterpart, human CTRP12 (hCTRP12) is also synthesized and secreted as both full-length (fCTRP12) and cleaved (gCTRP12) isoforms when expressed in heterologous HEK 293 cells (Fig. 9A). Importantly, conditioned medium containing hCTRP12 increased glucose uptake (Fig. 9B), as well as induced Akt phosphorylation (Fig. 9C) in 3T3-L1 adipocytes. These results suggest that human and mouse CTRP12 likely share similar function despite differences in tissue expression profiles between the two species. Future studies are needed to confirm the in vivo function of human CTRP12. Because many adipokines (e.g. adiponectin and RBP4) exhibit levels correlating with whole body insulin sensitivity and thus serve as useful biomarkers in disease states, it remains to be determined whether serum CTRP12 levels also reflect systemic insulin sensitivity in humans.

Injection of recombinant CTRP12 into WT mice increased serum insulin levels, partly accounting for the reduction of blood glucose. In contrast, administration of recombinant CTRP12 to obese mice (ob/ob and DIO) lowered blood glucose levels without altering serum insulin levels. Although the rea-
FIGURE 8. **CTRP12 promotes glucose uptake in adipocytes by activating PI3K-Akt signaling.**

A. CTRP12 (10 μg/ml) induces phosphorylation of IRS-1 (Tyr-612) in 3T3-L1 adipocytes. IRS1-P (10 μg/ml) increases glucose uptake in 3T3-L1 adipocytes. CTRP12 (10 μg/ml) increases glucose uptake in 3T3-L1 adipocytes, and the effect is nonadditive to that of insulin (10 nM). E and F, the increase of glucose uptake (E) and Akt phosphorylation (F) in 3T3-L1 adipocytes by CTRP12 (10 μg/ml) are inhibited by LY-294002 (50 μM). Ins, insulin (10 nM); LY, LY-294002; 12, recombinant CTRP12; Ctrl, control. All of the data are expressed as the means ± S.E. #, p < 0.001; **, p < 0.01; *, p < 0.05. α, p < 0.05 compared with control; β, not-significant compared with control; γ, p < 0.05 compared with insulin; δ, p < 0.05 compared with CTRP12.
玳for this is not clear, there are important and significant differences in insulin dynamics between WT and obese mice. In obese mice, insulin secretion is dramatically elevated to compensate for hyperglycemia resulting from insulin resistance in the peripheral tissues. In this hyperinsulinemic condition, it is likely that β-cell is unable to further increase its insulin secretion in response to acute stimulation by recombinant CTRP12. This may explain the differences in insulin secretion seen in WT versus obese (DIO and ob/ob) mice in response to recombinant protein injection.

Overexpressing CTRP12 in insulin-resistant obese mice (DIO and ob/ob) greatly enhanced insulin sensitivity, as reflected by multiple in vivo parameters: decreased fasting glucose, decreased HOMA-IR index, improved glucose tolerance, and decreased insulin secretion during GTT. Because insulin resistance is the major driver of insulin hypersecretion in the obese state, improvement in insulin sensitivity is predicted to lower serum insulin concentration, as shown in obese mice expressing CTRP12. This may explain why adenovirus-mediated expression of CTRP12 greatly reduced fed and fasting insulin levels in insulin-resistant ob/ob and DIO mice.

Because of the prevalence of insulin resistance and T2DM, there is great interest in discovering novel factors that can serve as therapeutic agents to restore insulin sensitivity and improve glycemia. Several secreted proteins, including adiponectin (32, 33), glucagon-like peptide-1 (GLP-1) (34), bone morphogenic protein-9 (bmp-9) (35), and fibroblast growth factor-21 (FGF-21) (36) are important metabolic regulators and have demonstrable glucose-lowering effects in vivo and thus are attractive targets for therapeutic development. Our functional studies suggest that CTRP12 may also be a potential candidate for the development of an anti-diabetic agent. Each of these metabolic regulators has a distinct mode of action. Adiponectin improves hepatic insulin action and enhances fatty acid oxidation in skeletal muscle by activating the AMPK signaling pathway (37, 38).

GLP-1 is an incretin hormone that activates the cAMP-dependent signaling pathway to promote glucose-dependent insulin secretion in pancreatic β-cells (34). FGF-21 activates multiple signaling pathways (e.g. Erk 1/2) to regulate insulin and glucagon secretion in the pancreas, improves hepatic insulin sensitivity, increases glucose uptake, and inhibits lipolysis in adipocytes (36).

In contrast to these endocrine factors, CTRP12 acts via insulin-dependent and independent pathways to modulate whole body glucose metabolism. On one hand, our in vivo studies clearly indicate that CTRP12 overexpression can improve insulin sensitivity in genetic (ob/ob) and diet-induced obese mouse models, in part by enhancing insulin signaling in adipose tissue and liver. On the other hand, our in vitro studies indicate that recombinant CTRP12 activates the PI3K-Akt signaling pathway, independent of insulin, to suppress gluconeogenesis in hepatocytes and promote glucose uptake in adipocytes. In vitro, CTRP12 and insulin act in an additive rather than synergistic manner. Thus, our studies suggest both insulin-dependent and independent mechanisms by which CTRP12 confers its beneficial metabolic effects in mice.

In summary, we provide evidence that CTRP12 is a novel adipokine with anti-diabetic actions that preferentially acts on adipose tissue and liver to control whole body glucose metabolism (Fig. 10). Further understanding of its mechanisms of action in physiologic and pathophysiologic states will likely provide novel insights and avenues to treat metabolic diseases such as T2DM.

Acknowledgments—We acknowledge the help of Hua Wang (Department of Physiology, Johns Hopkins University School of Medicine) with adenovirus injection. We thank Mehboob Hussain (Departments of Pediatrics and Medicine, Johns Hopkins University School of Medicine) for providing the INS-1 cell line. We thank Tamara Lotan (Department of Pathology, Johns Hopkins University School of Medicine) for help with adipose tissue histology.

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Anti-diabetic Actions of CTRP12


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C1q/TNF-related Protein-12 (CTRP12), a Novel Adipokine That Improves Insulin Sensitivity and Glycemic Control in Mouse Models of Obesity and Diabetes
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doi: 10.1074/jbc.M111.303651 originally published online January 24, 2012

Access the most updated version of this article at doi: 10.1074/jbc.M111.303651

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