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CTRP1 Protein Enhances Fatty Acid Oxidation via AMP-activated Protein Kinase (AMPK) Activation and Acetyl-CoA Carboxylase (ACC) Inhibition*

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Background: CTRP1 is an adiponectin paralog with poorly defined metabolic function.
Results: CTRP1 decreases diet-induced weight gain in mice by enhancing fatty acid oxidation and energy expenditure via activation of AMPK signaling.
Conclusion: CTRP1 is a novel regulator of lipid metabolism.
Significance: CTRP1 links adipose tissue to whole body energy balance.

We previously described the adipokine CTRP1, which has up-regulated expression following exposure to the anti-diabetic drug rosiglitazone and increased circulating levels in adiponectin-null mice (Wong, G. W., Krawczyk, S. A., Kitidis-Mitrokostas, C., Revett, T., Gimeno, R., and Lodish, H. F. (2008) *Biochem. J.* 416, 161–177). Although recombinant CTRP1 lowers blood glucose in mice, its physiological function, mechanisms of action, and roles in metabolic stress remain unknown. Here, we show that circulating levels of CTRP1 are strikingly reduced in diet-induced obese mice. Overexpressing CTRP1 in transgenic mice improved insulin sensitivity and decreased high-fat diet-induced weight gain. Reduced adiposity resulted from enhanced fatty acid oxidation and energy expenditure, effects mediated by AMP-activated protein kinase (AMPK). In skeletal muscle of transgenic mice, AMPKα and its downstream target, acetyl-CoA carboxylase (ACC), were hyperphosphorylated, indicative of AMPK activation and ACC inhibition. Inactivation of ACC promotes mitochondrial fat oxidation. Consistent with the direct effect of CTRP1 on AMPK signaling, recombinant CTRP1 administration acutely stimulated muscle AMPKα and ACC phosphorylation *in vivo*. In isolated soleus muscle, recombinant CTRP1 activated AMPK signaling to increase fatty acid oxidation *ex vivo*, an effect abrogated by an AMPK inhibitor. These results provide the first *in vivo* evidence that CTRP1 is a novel regulator of fatty acid metabolism.

Adipose tissue-derived hormones and cytokines (collectively termed adipokines) play important roles linking fat tissue to whole body energy balance (1). Some adipokines directly modulate tissue insulin sensitivity, as well as glucose and fatty acid metabolism, whereas other adipokines regulate metabolism indirectly through their effects on inflammatory processes. The health of adipose tissue in normal or disease states profoundly affects the array of secreted adipokines. For instance, adipose expression of the insulin-sensitizing hormone adiponectin is dramatically suppressed in obesity, but expression of retinol-binding protein (RBP)-4 and resistin, which promote insulin resistance, is significantly up-regulated (2–4).

To identify novel adipose tissue-derived secretory proteins with metabolic functions, we recently cloned a family of adiponectin paralogs on the basis of sequence homology to the globular C1q domain of adiponectin (5–9). These secreted proteins are designated as C1q/TNF-related proteins (CTRP1–10 and CTRP13). Mouse adiponectin and its CTRP[4] paralogs share only modest sequence identity at the functional globular domain, ranging from 30 to 51% at the amino acid level. However, adiponectin and CTRPs share multiple common biochemical features. First, CTRPs and adiponectin have similar domain structures; they are small secreted proteins (~30–40 kDa) belonging to the C1q family, possessing a signal peptide, a variable length N terminus with one or more conserved Cys residues, a collagen domain with variable numbers of Gly-*X*-*Y* repeats, and the signature C1q globular domain at the C terminus (10, 11). Second, CTRPs and adiponectin form trimers as their basic structural unit. Some CTRP trimers are further assembled into higher-order structures via intermolecular disulfide bonds mediated by the conserved N-terminal Cys residues, analogous to adiponectin (6, 7). Third, adiponectin and CTRPs contain similar types of posttranslational modifications, including proline hydroxylation and lysine glycosylation (6, 12); for adiponectin, these posttranslational modifications affect its *in vivo* function and bioactivity (12, 13). Fourth, adiponectin and CTRPs circulate in plasma, with levels affected by sex, met-

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[4] The abbreviations used are: CTRP, C1q/TNF-related protein; AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; DIO, diet-induced obese; Tg, transgenic; RER, respiratory exchange ratio; LF, low-fat; HF, high-fat; GTT, glucose tolerance test; AICAR, aminoimidazole carboxamide ribonucleotide; Bis-Tris, 2-(bis(2-hydroxyethyl)amino)-2-(hydroxymethyl)propane-1,3-diol.

abolic state, and genetic background of the animal (6–9). However, unlike adiponectin, which is produced primarily by adipocytes, CTRPs come predominantly from adipose tissue but are more widely expressed (5–9). Although the metabolic roles of adiponectin are well described (14, 15), those of CTRPs remain poorly understood and are being actively investigated.

Despite high circulating levels (10–30 μg/ml) and well documented anti-diabetic, anti-atherogenic, and anti-inflammatory properties in various animal models, adiponectin is not an essential gene; targeted disruption in mice results in surprisingly mild metabolic alterations (16–19). Although enhanced leptin sensitivity is proposed to account for the mild metabolic phenotypes of adiponectin-null mice (20), other secreted factor(s) such as the CTRPs may partially compensate for the loss of adiponectin (21). Indeed, CTRP1 may be one such candidate. First, circulating levels of CTRP1 increase ~2-fold in adiponectin-null mice (7). Second, administration of the anti-diabetic drug rosiglitazone (a peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist) up-regulates expression of CTRP1 mRNA in mouse adipose tissue (7). This finding suggests that CTRP1 may mediate some of the beneficial metabolic effects of rosiglitazone *in vivo*, as has been shown for adiponectin (19, 22). Third, injection of recombinant CTRP1 into mice acutely lowers blood glucose (7).

Beyond its glucose-lowering effect in mice, nothing is known about the mechanisms by which CTRP1 exerts its metabolic function *in vivo* or its potential role in diet-induced metabolic stress. Here, we provide the first mechanistic insight into the metabolic function of CTRP1 *in vivo* and establish its importance as a novel metabolic regulator.

MATERIALS AND METHODS

Antibodies and Chemicals—Mouse monoclonal anti-FLAG M2 antibody was obtained from Sigma, and rat monoclonal anti-HA (clone 3F10) antibody was obtained from Roche Applied Science. Rabbit anti-CTRP1 antibody was obtained from AnaSpec. Rabbit antibodies that recognize phospho-Akt (Thr-308), phospho-AMPKα (Thr-172), phospho-ACC (Ser-79), and total Akt, AMPKα, and ACC were obtained from Cell Signaling Technology. AMPK activator (AICAR, aminoimidazole carboxamide ribonucleotide) (23) and inhibitor (Compound C) (24) were obtained from Calbiochem.

Animals—Six-week-old, male wild-type and CTRP1 transgenic mice (both C57BL/6) were housed in polycarbonate cages on a 12-h light-dark photocycle and had access to water *ad libitum* throughout the study period. Unless otherwise noted, mice were fed *ad libitum* with standard laboratory chow diet (chow; 13.5% kcal from fat; number 5001, LabDiet, PMI Nutrition International, St. Louis, MO) or high-fat (HF) diet (55% kcal from fat, Harlan Teklad; TD.93075.PWD). HF diet was provided for a period of 12–18 weeks, starting at 4–5 weeks of age, to make mice diet-induced obese (DIO). Blood samples were collected for serum analysis. Tissues were collected, snap-frozen in liquid nitrogen, and kept at −80 °C. All animal protocols were approved by the Institutional Animal Care and Use Committee of Johns Hopkins University School of Medicine.

Generation of CTRP1 Transgenic Mice—C-terminal HA epitope (YPYDVPDYA)-tagged CTRP1 was cloned into the EcoRI and XhoI site of pCAGGS vector (25). Expression of the CTRP1 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 the resulting DNA fragments (~3.5 and 2.5 kb) were separated on a 1% agarose gel. The ~3.5-kb linear DNA fragment containing the CAG promoter and enhancer, CTRP1 transgene, and rabbit β-globin poly(A) 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) were obtained. One mouse line with detectable HA-tagged CTRP1 protein in the serum was maintained and expanded for phenotypic analysis. Transgenic (Tg) mice are fertile and were born at the expected Mendelian ratio. No gross abnormality was observed in these animals.

Intraperitoneal Glucose and Insulin Tolerance Tests—Separate cohorts of 8–10 Tg and aged-matched male WT control littermates were injected with glucose (1 g/kg) or insulin (0.8 units/kg for chow-fed mice, 1.2 units/kg for HF diet-fed mice). Animals were fasted overnight (16 h) prior to the glucose tolerance test. For the insulin tolerance test, food was removed 2 h prior to insulin injection. Serum samples were collected at the indicated time points shown under "Results." Insulin and glucose tolerance tests were performed at 17–18 weeks of age.

Protein Purification—Recombinant full-length mouse CTRP1, containing a C-terminal FLAG tag epitope (DYKDDDDK), was produced in mammalian cells as described previously (8). Briefly, HEK 293 cells (GripTite^{TM} cells, Invitrogen) were cultured in DMEM containing 10% (v/v) bovine calf serum supplemented with antibiotics. Transfections were performed in HEK 293 cells using the calcium phosphate method (26). At 48 h after transfection, media were replaced with serum-free Opti-MEM (Invitrogen) supplemented with vitamin C (0.1 mg/ml). Supernatants were collected three times, every 48 h, pooled, and purified using an anti-FLAG affinity gel according to the manufacturer's protocol (Sigma) and then eluted with 150 μg/ml FLAG peptide (Sigma). Purified proteins were dialyzed against 20 mM Hepes buffer (pH 8.0) containing 135 mM NaCl in a 10-kDa cut-off Slide-A-Lyzer dialysis cassette (Pierce). Protein concentration was determined using a Coomassie Plus protein assay reagent (Thermo Scientific).

Recombinant Protein Injection—Experiments were carried out as described previously (7, 8). Briefly, food was removed in the morning (around 8–9 a.m.), 2 h prior to recombinant protein injection; drinking water was supplied for the duration of the experiment. Recombinant CTRP1 (2 μg/g of body weight) or the equivalent volume of vehicle buffer (20 mM Hepes (pH 8.0) containing 135 mM NaCl) was injected intraperitoneally into 10-week-old C57BL/6 mice ($n = 8$). Ninety minutes later, mice were sacrificed, and triceps surae muscles were immediately isolated and snap-frozen in liquid nitrogen. Homogenized muscle cell lysates were prepared in lysis buffer (T-PER, Thermo Scientific) containing protease and phosphatase inhibitor mixtures (Sigma).

Fatty Acid Oxidation—Experiments were carried out as described previously (27). Briefly, soleus muscles were isolated and preincubated for 20 min at 37 °C in glass tubes with Krebs-

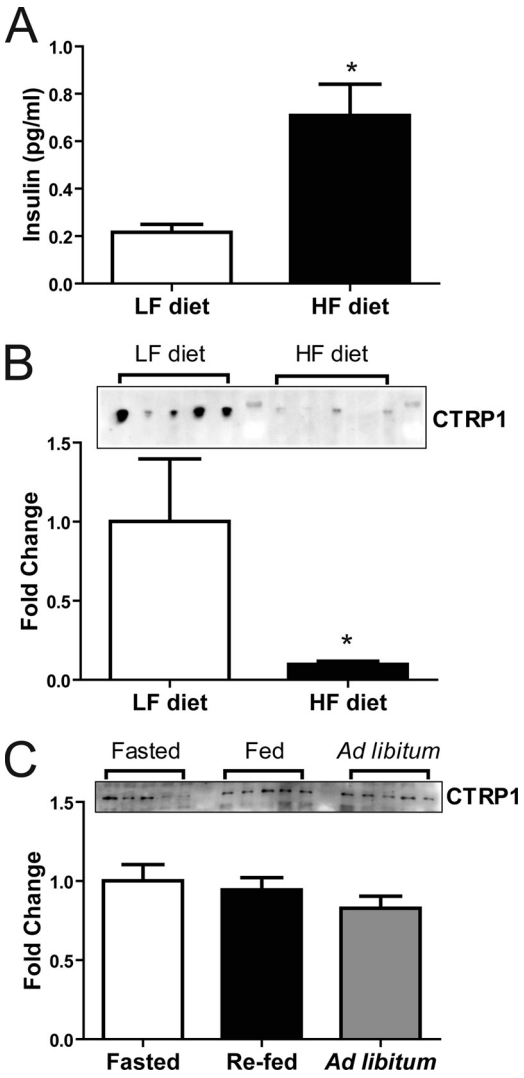

FIGURE 1. **Reduction in circulating CTRP1 levels in insulin-resistant DIO mice.** *A*, fasting serum insulin concentrations in mice on an HF diet (60% kcal from fat) when compared with mice on an LF diet (10% kcal from fat) ($n = 10$). *B*, quantitative immunoblot analysis of serum CTRP1 levels in mice fed an HF or an LF diet ($n = 10$). *C*, quantitative immunoblot analysis of serum CTRP1 levels in 12-week-old chow-fed male mice under fasted, fasted and refed, or *ad libitum* conditions. Values shown are mean -fold change ± S.E. relative to LF diet group. *, $p < 0.05$.

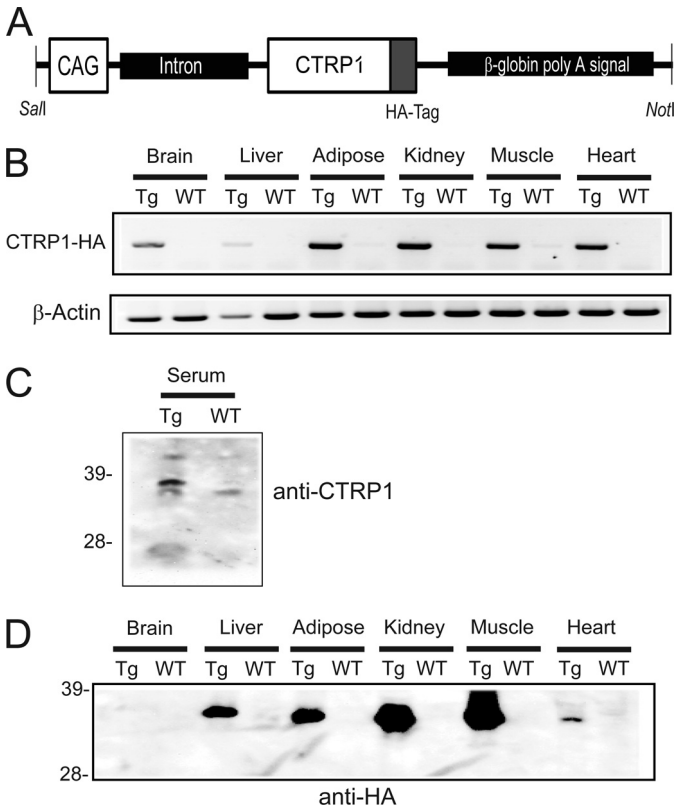

FIGURE 2. **Generation of CTRP1 transgenic mouse line.** *A*, schematic of CTRP1 transgenic construct. HA-tagged CTRP1 transgene is driven by the ubiquitous CAG promoter. *B*, semiquantitative RT-PCR analysis of CTRP1-HA transgene and β-actin control expression in mouse tissues. *C*, immunoblot analysis of serum CTRP1 in WT and Tg mice using a CTRP1-specific antibody. *D*, immunoblot analysis for the presence of CTRP1-HA protein in mouse tissues using an anti-HA antibody.

Henseleit buffer (Sigma K3753) containing 1 μg/ml insulin, 2% fatty acid-free BSA, and recombinant CTRP1 (5 μg/ml), vehicle control (v/v), or AICAR (2 mM) in the presence or absence of Compound C (10 μM). For fatty acid oxidation studies, 0.2 μCi/ml [1-^{14}C]oleic acid (PerkinElmer Life Sciences) was added to the medium and incubated for 60 min. Glass tubes were sealed with a rubber septum from which a center well carrying a piece of Whatman filter paper was suspended. At the end of a 60-min incubation, the medium was acidified with 35% perchloric acid, and the filter paper was wetted with 0.45 ml of 3 M NaOH and shaken at 37 °C. After a 90-min collection period, the filter paper was removed from the center well and transferred to liquid scintillation vial, and the amount of ^{14}C radioactivity was determined using an LS6000SC instrument (Beckman Coulter).

Serum and Blood Chemistry Analysis—Mouse serum samples were harvested by tail bleed and separated using Microvette® CB 300 (The SARSTEDT Group). Glucose concentration was determined at the time of collection with a glucometer (BD Biosciences). Serum/tissue triglyceride (Thermo Fisher), non-esterified free fatty acid (Wako), insulin, leptin, TNF-α, plasminogen activator inhibitor (PAI-1), adiponectin, and resistin (Millipore) were determined using commercially available kits. Total cholesterol was measured at the Molecular and Comparative Pathobiology phenotyping core facility at the Johns Hopkins University School of Medicine.

Body Composition Analysis—Body composition of Tg and WT mice was determined using a whole body NMR instrument (EchoMRI, Echo Medical Systems LLC, Waco, TX) housed at the Molecular and Comparative Pathobiology phenotyping core facility at the Johns Hopkins University School of Medicine. EcoMRI analysis provided values for fat mass, lean mass, and water content.

Indirect Calorimetry—CTRP1 Tg mice and WT controls ($n = 7–8$/group) were used for simultaneous assessments of daily body weight change, energy intake (corrected for spillage), and whole body metabolic profile in an open-flow indirect calorimeter (Oxymax, Columbus Instruments). Mice were chow-fed lean mice (16 weeks old) or DIO mice on an HF diet, in separate studies. Data were collected for 3 days to confirm acclimation to the calorimetry chambers (stable body weights and food intakes), and data from the 4th day in the Oxymax were

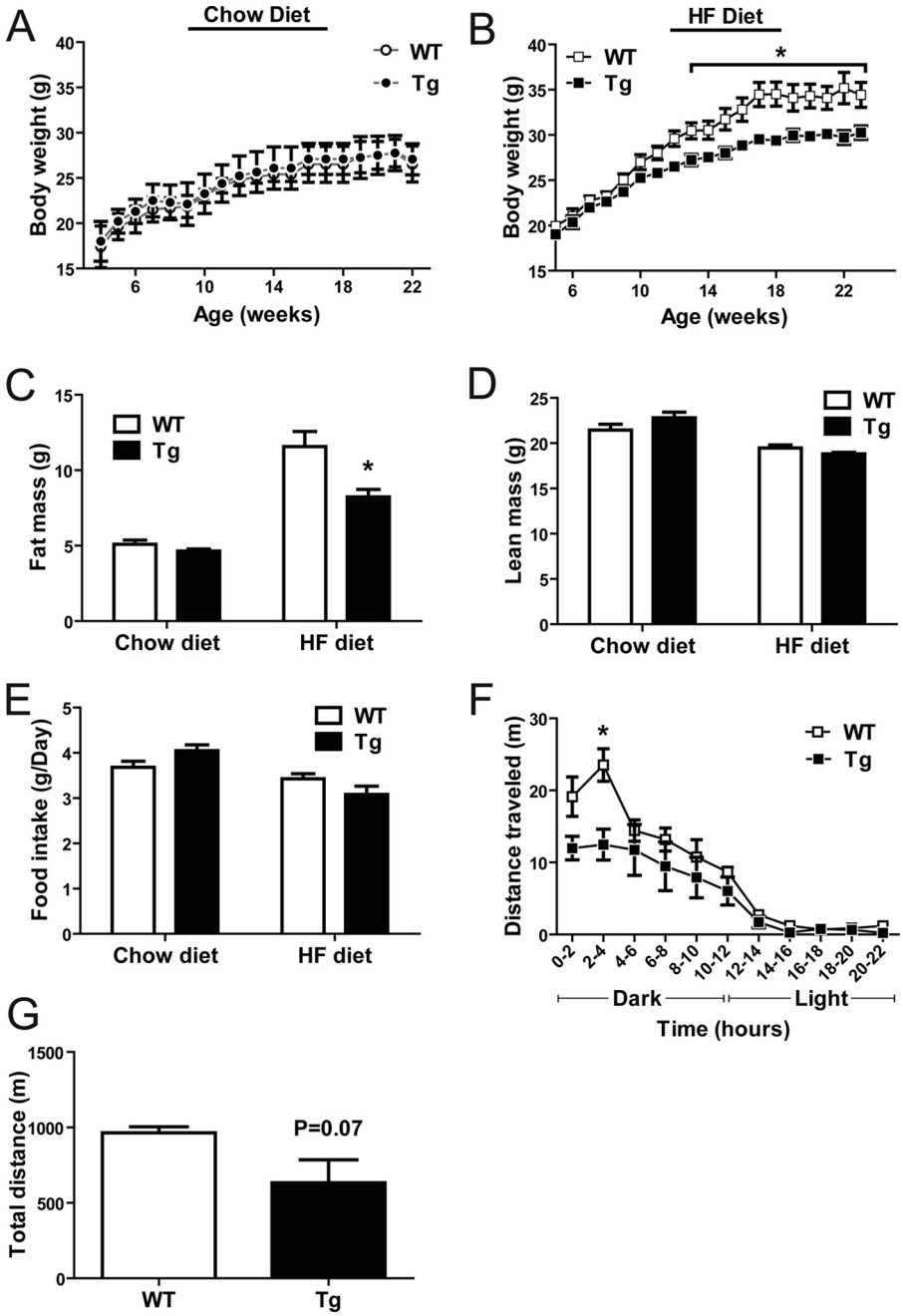

FIGURE 3. **Reduced adiposity in transgenic mice on a high-fat diet.** *A*, body weight gain over time between WT and Tg male mice on a standard chow diet. *B*, body weight gain over time between WT and Tg male mice on an HF diet. *C* and *D*, fat mass (*C*) and lean mass (*D*) in WT and Tg mice on a chow or an HF diet were determined using an NMR instrument (EchoMRI). *E*, food intake analysis in WT and Tg mice on a chow or an HF diet. *F*, 24-h ambulatory activity of WT and Tg mice on an HF diet. Data were compiled into 2-h segments. *G*, total voluntary physical activity in 24 h between WT and Tg mice on an HF diet. Data are reported as mean -fold change ± S.E. of 8–10 mice/group. *, $p < 0.05$ *versus* WT.

analyzed. Rates of oxygen consumption (V_{O2}, ml/kg/h) and carbon dioxide production (V_{CO2}) were measured for each chamber every 15 min throughout the studies. Respiratory exchange ratio (RER = V_{CO2}/V_{O2}) was calculated by Oxymax software (version 4.02) to estimate relative oxidation of carbohydrate (RER = 1.0) *versus* fat (RER approaching 0.7), not accounting for protein oxidation. Energy expenditure was calculated as EE = $V_{O2} \times (3.815 + (1.232 \times \text{RER}))$ (28) and normalized for body mass (kcal/kg/h). Average metabolic values were calculated per subject and averaged across subjects for statistical analysis by Student's *t* test, with $p \leq 0.05$ indicating significant differences between groups.

Semiquantitative PCR Analysis—Total RNAs from mouse tissues were isolated with TRIzol® (Invitrogen). Two micrograms of total RNA was reverse-transcribed using SuperScript III (Invitrogen). Thirty-cycle PCR was carried out using Platinum® Pfx DNA polymerase (Invitrogen). Cycling conditions were as follows: 15 s of denaturation at 95 °C, 15 s of primer annealing at 60 °C, and 45 s of primer extension at 72 °C. Primers used were as follows: CTRP1-HA, forward, 5′-CGGCAAAATAGGTTCTACAG-

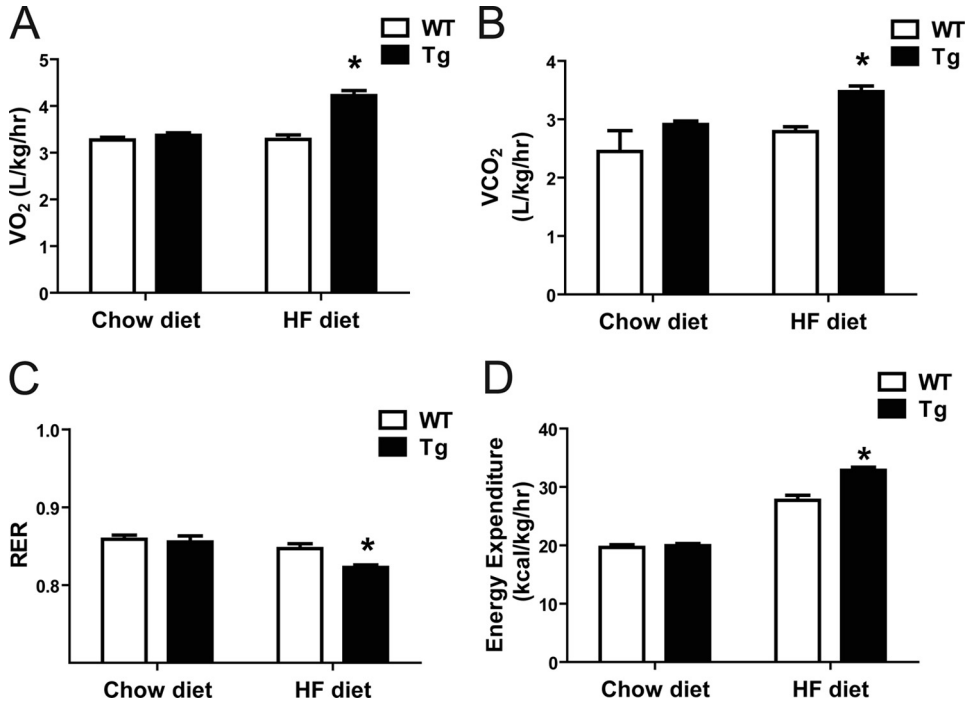

FIGURE 4. **Enhanced fatty acid oxidation and energy expenditure in CTRP1 transgenic mice.** *A–D*, oxygen consumption (V_{CO2}; *A*), carbon dioxide release (V_{CO2}; *B*), respiratory exchange ratio (RER = V_{CO2}/V_{O2}; *C*), and energy expenditure (*D*) of WT and Tg mice on a chow or an HF diet were determined by indirect calorimetry analysis. Energy expenditure was calculated as kcal per subject and then normalized to lean body mass in kg. Data are reported as mean ± S.E. of 8–10 mice/group. *, $p < 0.05$ *versus* WT.

GTC-3′, and reverse, 5′-TCAAGCGTAGTCTGGGACGTCGTATGG-3′; β-actin, forward, 5′-CGTGACATTAAGGAGAAGCTGTGC-3′, and reverse, 5′-CTCAGGAGGAGCAATGATCTTGAT-3′.

Immunoblot Analysis—Serum samples were diluted 1:20 in SDS loading buffer (50 mM Tris-HCl, pH 7.4, 2% SDS w/v, 6% glycerol w/v, 1% 2-mercaptoethanol v/v, and 0.01% bromphenol blue w/v) and were separated on 10% Bis-Tris NuPAGE gel (Invitrogen). Each well was loaded with an equivalent of 1 μl of serum. For skeletal muscle lysates, 10 μg of protein was loaded and separated on a 10% Bis-Tris NuPAGE gel. Fractionated proteins were then transferred to Protran BA83 nitrocellulose membranes (Whatman), blocked in 2% nonfat milk for 1 h, and probed with primary antibodies in the presence of 2% nonfat milk overnight. Immunoblots were washed three times (10 min each) in PBS containing 0.1% Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences) (1:5000) for 1 h. Blots were washed three times (10 min each) in PBS containing 0.1% Tween 20, developed in ECL reagent (Millipore) for 2–5 min, and visualized with MultiImage III FluorChem® Q (AlphaInnotech). Quantifications of signal intensity were performed using AlphaView software (AlphaInnotech).

Statistical Analysis—All comparisons between CTRP1 Tg and WT littermate controls were made within diet groups only. Results are expressed as means ± S.E. Statistical analysis was performed with Prism 5 software (GraphPad). Blood chemistry data were analyzed with Student's *t* tests between CTRP1 Tg and WT mice. Repeated measures analyses of variance were performed on body weights and serum glucose and insulin measurements during various tolerance tests. Values were considered to be significant at $p < 0.05$.

TABLE 1
Adipokine and blood chemistry analysis

Overnight fasted sera were collected from 22-week-old WT and Tg mice ($n = 8–10$) fed an HF diet for a period of 18 weeks. NEFA, non-esterified free fatty acid; NS, not significant.

	WT	Tg	*p* value
Chow diet			
Insulin (ng/ml)	1.01 ± 0.15	1.15 ± 0.13	NS
Glucose (mg/dl)	80.8 ± 4.6	76.8 ± 2.0	NS
Leptin (ng/ml)	1.15 ± 0.23	0.99 ± 0.19	NS
Adiponectin (μg/ml)	17.9 ± 0.9	18.2 ± 2.1	NS
TNF-α (pg/ml)	14 ± 0.8	15 ± 0.4	NS
PAI-1 (total; ng/ml)	1.26 ± 0.24	1.19 ± 0.13	NS
Resistin (ng/ml)	1.25 ± 0.12	1.08 ± 0.20	NS
NEFA (meq/liter)	0.2 ± 0.004	0.3 ± 0.005	NS
Serum triglycerides (mg/dl)	43.2 ± 2.2	50.4 ± 2.7	NS
HF diet			
Insulin (ng/ml)	1.75 ± 0.21	1.64 ± 0.16	NS
Glucose (mg/dl)	188 ± 5.1	187 ± 6.6	NS
Leptin (ng/ml)	8.02 ± 2.8	11.9 ± 5.7	NS
Adiponectin (μg/ml)	14.0 ± 1.0	10.1 ± 0.5	<0.05
TNF-α (pg/ml)	15 ± 0.3	14 ± 0.2	NS
PAI-1 (total; pg/ml)	2.24 ± 0.24	2.89 ± 0.64	NS
Resistin (pg/ml)	2.13 ± 0.16	2.15 ± 0.41	NS
NEFA (meq/liter)	0.2 ± 0.004	0.2 ± 0.005	NS
Serum triglycerides (mg/dl)	70.6 ± 5.6	78.2 ± 4.7	NS
Liver triglycerides (μg/mg of tissue)	50.5 ± 3.2	53.2 ± 3.8	NS
Muscle triglycerides (μg/mg of tissue)	8.2 ± 0.9	8.0 ± 0.4	NS

RESULTS

Reduction in Circulating Levels of CTRP1 in DIO Mice—To test whether serum CTRP1 levels are responsive to alterations in whole body energy balance, we examined the serum levels of this protein in obese mice. Mice fed an HF diet for 12 weeks had much higher levels of fasting insulin when compared with mice on an isocaloric matched low-fat (LF) diet (Fig. 1*A*), indicating diet-induced insulin resistance in DIO mice. Correspondingly, we also observed ~90% reduction in the circulating levels of CTRP1 in these animals (Fig. 1*B*), indicating that diet-induced

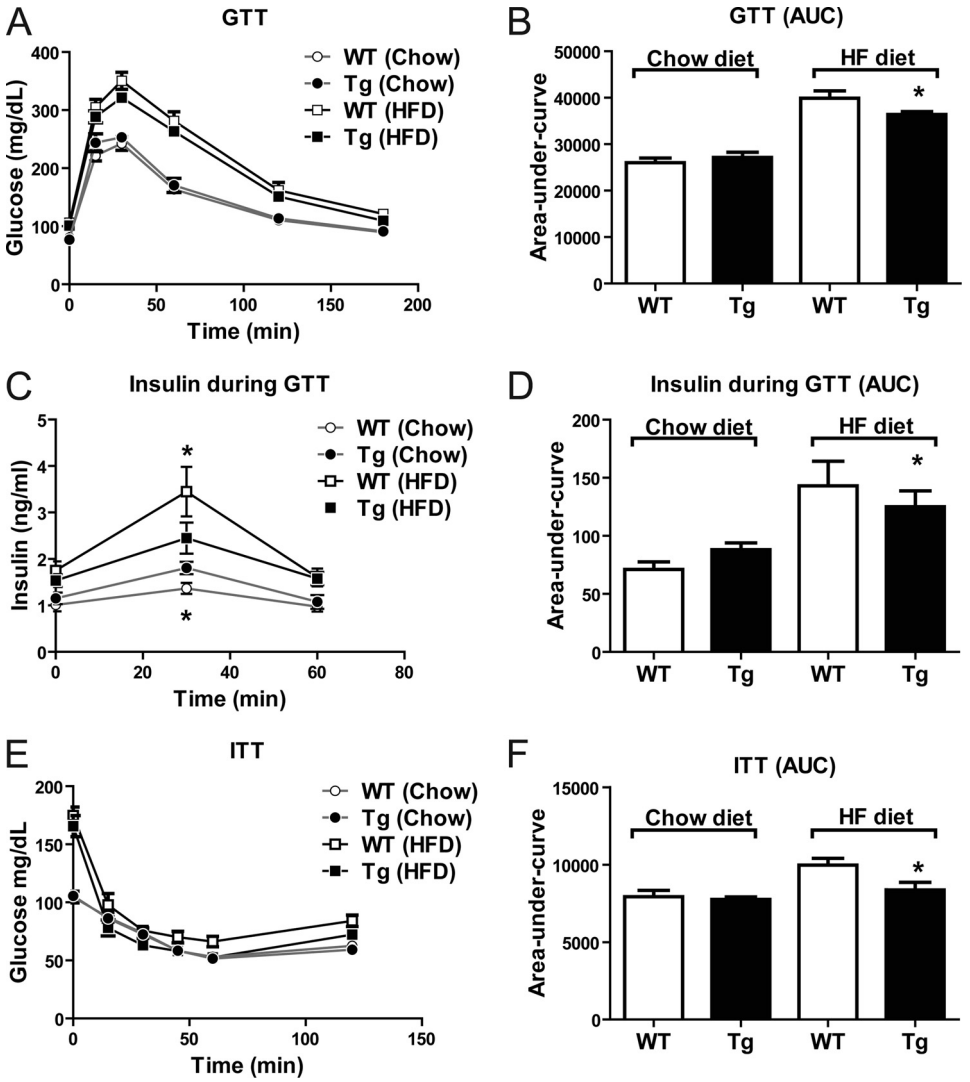

FIGURE 5. **Improved insulin sensitivity in CTRP1 transgenic mice.** *A*, intraperitoneal GTT. *B*, quantification of the cumulative glucose clearance in GTT by integration of the area under the curve (*AUC*). *C*, insulin levels during the course of GTT. *D*, quantification of the cumulative insulin release in GTT by integration of the area under the curve. *E*, insulin tolerance test (*ITT*). *F*, quantification of cumulative glucose clearance in insulin tolerance test by integration of the area under the curve. Values are means ± S.E. of 8–10 mice/group. *, $p < 0.05$ *versus* WT.

metabolic stress affects serum levels of CTRP1. Next, we examined whether short-term changes in nutritional states acutely affect circulating levels of CTRP1. Mice that were fasted overnight, fasted and refed for 2 h or that had access to food *ad libitum* did not show acute changes in circulating levels of CTRP1 (Fig. 1*C*).

Generation of CTRP1 Tg Mouse Line—To address the metabolic function of CTRP1, we generated a Tg mouse model overexpressing HA epitope-tagged CTRP1. In mice, CTRP1 mRNA is expressed predominantly by the stromal vascular fraction of adipose tissue, but also by other tissues, albeit at lower levels (7). For this reason, expression of CTRP1 transgene was driven by a ubiquitous promoter (Fig. 2*A*). In the Tg mouse line, the CTRP1 transgene was expressed in adipose tissue, kidney, skeletal muscle, and heart, and lower levels were also detected in the brain (Fig. 2*B*). As expected, the Tg mouse line has higher circulating levels of CTRP1, with an ~2-fold increase over baseline serum levels detected in wild-type mice (Fig. 2*C*). Although mRNA expression of CTRP1 transgene was comparable in mouse tissues, we observed a much higher level of CTRP1-HA protein in skeletal muscle when compared with other tissues (Fig. 2*D*). Because CTRP1 is a secreted protein that circulates in plasma and our present study identified skeletal muscle as a major target tissue of CTRP1, the substantially higher CTRP1 protein level detected in skeletal muscle likely reflects greater accumulation of CTRP1 protein at the site of action.

CTRP1 Tg Mice Show Reduced Weight Gain in Response to an HF Diet—On a standard laboratory chow diet, body weight gain over time was indistinguishable between WT and Tg male mice (Fig. 3*A*). However, when challenged with an HF diet, Tg mice gained significantly less body weight over time (Fig. 3*B*). By 23 weeks of age, Tg mice (34.4 ± 1.5 g) weighed 12% less than WT littermate controls (30.3 ± 0.9 g). Body composition analysis using NMR showed that Tg mice had significantly less fat mass when compared with WT controls (Fig. 3*C*; 8.2 ± 0.6 *versus* 11.6 ± 1.1 g), thus accounting for lower body weights in Tg mice. In contrast, the two groups of mice had similar amounts

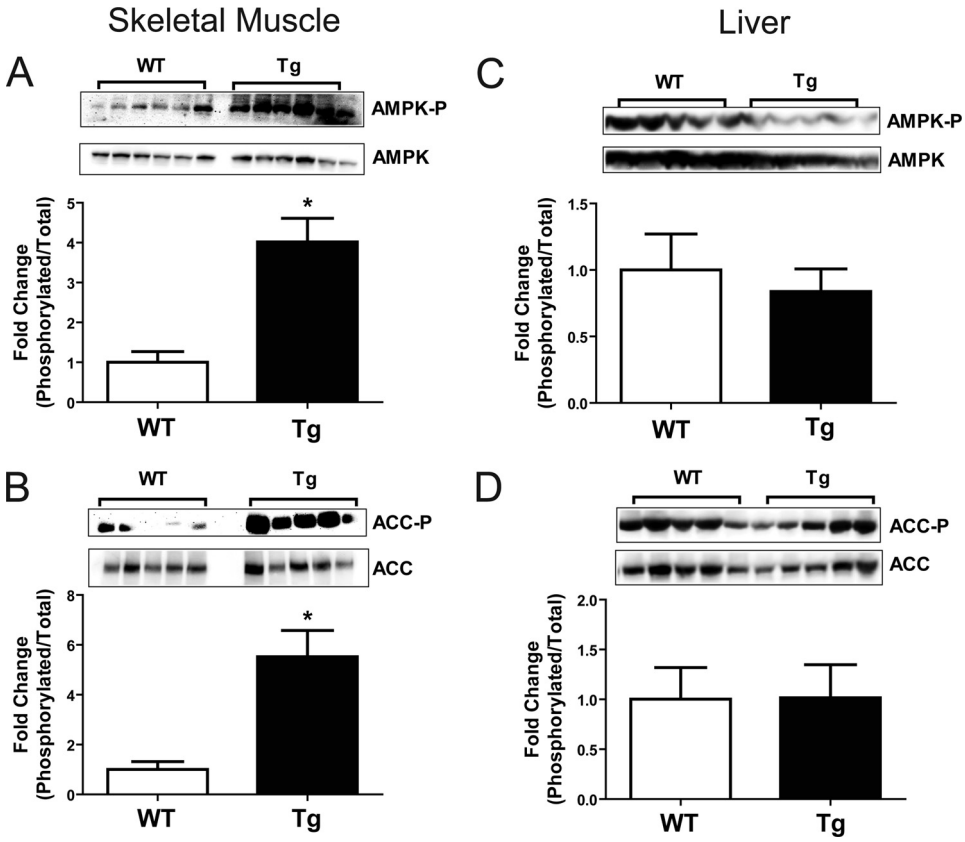

FIGURE 6. **Hyperphosphorylation of AMPKα and ACC in skeletal muscle of CTRP1 transgenic mice.** *A* and *B*, quantitative immunoblot analysis of skeletal muscle AMPKα (Thr-172) (*A*) and ACC (Ser-79) (*B*) phosphorylation in WT and Tg mice on an HF diet ($n = 8–10$ mice/group). *AMPK-P*, AMPK phosphorylation; *ACC-P*, ACC phosphorylation. *C* and *D*, quantitative immunoblot analysis of liver AMPKα (*C*) and ACC (*D*) phosphorylation in WT and Tg mice on an HF diet ($n = 8–10$). Phosphorylated protein levels were normalized to total protein levels. All data are reported as mean -fold change ± S.E. *, $p < 0.05$ *versus* WT.

of lean mass (Fig. 3*D*). Reduced adiposity in response to HF diet could result from differences in food intake or voluntary physical activity levels. However, no differences in food intake were observed between Tg and WT mice (Fig. 3*E*). Further, when ambulatory activity was measured, Tg mice were found to be slightly less active during the night photocycle (Fig. 3*F*). Based on total distance traveled, there appeared to be a trend toward a reduction in voluntary physical activity in Tg when compared with WT mice (Fig. 3*G*), indicative of decreased energy expenditure by physical activity.

Reduced Fat Mass in Tg Mice Is due to Enhanced Fatty Acid Oxidation and Energy Expenditure—To determine the consequences of CTRP1 overexpression on whole body energy balance, we performed indirect calorimetry analyses. On a chow diet, Tg and WT male mice had similar V_{O2}, V_{CO2}, RER, and energy expenditure. In contrast, Tg mice on an HF diet had increased oxygen consumption (Fig. 4*A*) and increased carbon dioxide production (Fig. 4*B*) relative to WT mice, indicating enhanced metabolism. Further analysis showed that Tg mice had a lower RER (0.847 ± 0.006 *versus* 0.823 ± 0.003; Fig. 4*C*), indicating a shift in the preferential utilization of fat as a substrate for energy production. Due to enhancement of fatty acid oxidation, Tg mice had an overall increase (~28%) in whole body energy expenditure when compared with WT mice (Fig. 4*D*).

Adipokine and Blood Chemistry Profiles of WT and Tg Mice—Serum metabolic parameters were measured in Tg and WT mice on a chow or HF diet (Table 1). Under both diet conditions, Tg and WT mice had similar fasting serum levels of cholesterol, triglycerides, non-esterified free fatty acid, insulin, glucose, TNF-α, leptin, resistin, and plasminogen activator inhibitor (PAI-1). In contrast, there was a significant reduction (~30%) in serum adiponectin levels in Tg mice on an HF diet when compared with WT controls. However, real-time PCR analysis did not reveal any statistically significant differences in adiponectin mRNA expression in the adipose tissue of WT and Tg mice (data not shown).

CTRP1 Tg Mice Are More Insulin-sensitive—When Tg and WT mice on an HF diet were fasted overnight, no differences were observed in their fasting blood glucose concentrations (Table 1). However, as revealed by the area under the curve in the glucose tolerance test (GTT), a modest overall improvement in cumulative glucose disposal was observed in Tg relative to WT mice (Fig. 5, *A* and *B*). The rate of glucose clearance in peripheral tissues is dependent on the magnitude of insulin release in response to glucose challenge and tissue sensitivity to insulin (29). Despite greater cumulative glucose disposal, the magnitude of insulin release in response to glucose challenge was smaller in Tg mice relative to WT controls (Fig. 5, *C* and *D*), indicating modest improvements in insulin sensitivity in peripheral tissues. Consistent with this, Tg mice on an HF diet showed an overall greater cumulative glucose clearance in response to insulin administration in the insulin tolerance test (Fig. 5, *E* and *F*).

Activation of AMPK and Inhibition of ACC in Skeletal Muscle of Tg Mice—Phosphorylation at Thr-172 on the catalytic α-subunit of AMPK activates this enzyme (30–32). Activated AMPK phosphorylates Ser-79 on ACC and inactivates the enzyme, leading to reduced intracellular malonyl-CoA concentration (33–35). Malonyl-CoA is an allosteric inhibitor of carnitine palmitoyl transferase 1 (CPT1), the rate-limiting enzyme catalyzing the import of fatty acyl-CoA into mitochondria for β-oxidation (36, 37). Thus, AMPKα phosphorylation and activation increase fatty acid oxidation. Consistent with enhanced fat oxidation seen in Tg mice on an HF diet (Fig. 4, *A* and *C*), we observed hyperphosphorylation of AMPKα (Thr-172) and its downstream target, ACC (Ser-79), in skeletal muscle of these animals (Fig. 6, *A* and *B*), indicating AMPK activation and ACC inhibition. Activation of AMPK signaling appears to be specific; other signaling pathways such as protein kinase B/Akt, p42/44 MAPK, and JAK/STAT were not activated in skeletal muscle of Tg mice (data not shown). In contrast to skeletal muscle, AMPKα and ACC were not hyperphosphorylated in the liver of Tg mice (Fig. 6, *C* and *D*), suggesting that AMPK activation mediated by CTRP1 is muscle-specific.

Recombinant CTRP1 Acutely Induced AMPKα and ACC Phosphorylation in Skeletal Muscle—Activation of AMPK signaling in the skeletal muscle of Tg mice could be a direct effect of CTRP1 or a secondary effect resulting from chronic overexpression of CTRP1. To demonstrate that CTRP1 can acutely activate AMPK signaling *in vivo*, we injected recombinant CTRP1 or vehicle control into wild-type C57BL/6 male mice and harvested the skeletal muscles at 90 min after injection. Western blot analysis showed enhanced phosphorylation of AMPKα (Thr-172) and ACC (Ser-79) in muscle samples derived from CTRP1-injected mice relative to vehicle-injected controls (Fig. 7). The acute effect mediated by recombinant CTRP1 was only observed in skeletal muscle; we did not observe any increase in AMPKα and ACC phosphorylation in the liver of CTRP1-injected mice relative to vehicle-injected controls (data not shown). This is consistent with the observed chronic hyperphosphorylation of both AMPKα and ACC in skeletal muscle, but not liver, of Tg mice (Fig. 6).

CTRP1 Acts via AMPK to Increase Fat Oxidation in Isolated Soleus Muscle—In isolated soleus, a predominantly slow-twitch muscle, recombinant CTRP1 acutely stimulated AMPKα phosphorylation (Fig. 8*A*) and enhanced fatty acid oxidation (Fig. 8*C*) in a manner similar to soleus muscle stimulated with the AMPK activator AICAR (Fig. 8*B*). Although AMPK inhibitor, Compound C, alone had no effect on isolated soleus muscle (Fig. 8*D*), it abrogated the ability of recombinant CTRP1 to enhance fatty acid oxidation (Fig. 8*E*). These results confirm that CTRP1 directly regulates fatty acid oxidation in skeletal muscle via the activation of AMPK.

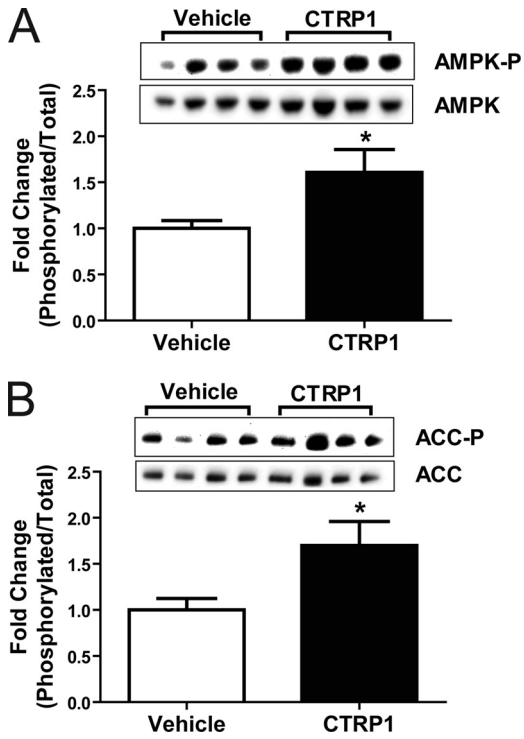

FIGURE 7. **Recombinant CTRP1 acutely induces AMPKα and ACC phosphorylation in skeletal muscle of wild-type mice.** *A* and *B*, quantitative immunoblot analysis of skeletal muscle AMPKα (Thr-172) (*A*) and ACC (Ser-79) (*B*) phosphorylation in wild-type male mice injected with vehicle or recombinant CTRP1 (2 μg/g of body weight; $n = 8$). Triceps surae muscles were harvested 90 min after injection. Phosphorylated protein levels were normalized to total protein levels. All data are reported as mean -fold change ± S.E. *, $p < 0.05$ *versus* vehicle. *AMPK-P*, AMPK phosphorylation; *ACC-P*, ACC phosphorylation.

DISCUSSION

We establish here the novel metabolic function and mechanism of action of CTRP1. Using multiple approaches involving *in vivo* recombinant protein administration, transgenic overexpression, and *ex vivo* functional study on isolated soleus, we showed that skeletal muscle is a direct target tissue of CTRP1. Further, we demonstrated that CTRP1 acts via AMPK in muscle to enhance fatty acid oxidation. A serine/threonine protein kinase, AMPK is an evolutionarily conserved heterotrimeric energy sensor activated by the rise of cellular AMP concentrations or by hormonal stimulation involving upstream AMPK kinases (*e.g.* LKB1) (38). Many signaling molecules and metabolic enzymes are regulated by AMPK phosphorylation. Activated AMPK shuts off anabolic processes that consume ATP while turning on catabolic processes that generate ATP. By activating AMPK, CTRP1 promotes whole body energy expenditure, as seen in CTRP1 Tg mice. Because skeletal muscle mass accounts for 20–25% of total body weight, a modest increase in muscle fat oxidation will result in significant energy expenditure over time. Consistent with this, CTRP1 Tg mice accumulated less fat mass when compared with WT controls on a high-fat diet.

Circulating levels of CTRP1 are dramatically reduced in DIO mice. Forced expression of CTRP1 prevented the reduction of serum CTRP1 levels in Tg mice subjected to HF diet feeding. This, in turn, partially ameliorated the HF diet-induced weight gain in Tg mice relative to littermate controls. This finding may partly explain why the beneficial metabolic effects of CTRP1 are only revealed under conditions of metabolic stress, wherein existing homeostatic mechanisms are dysregulated.

It appears that in Tg mice, the primary mechanism by which CTRP1 enhances fat oxidation in skeletal muscle is regulating AMPK and ACC activity via protein phosphorylation; we did not observe any long-term changes in expression of metabolic

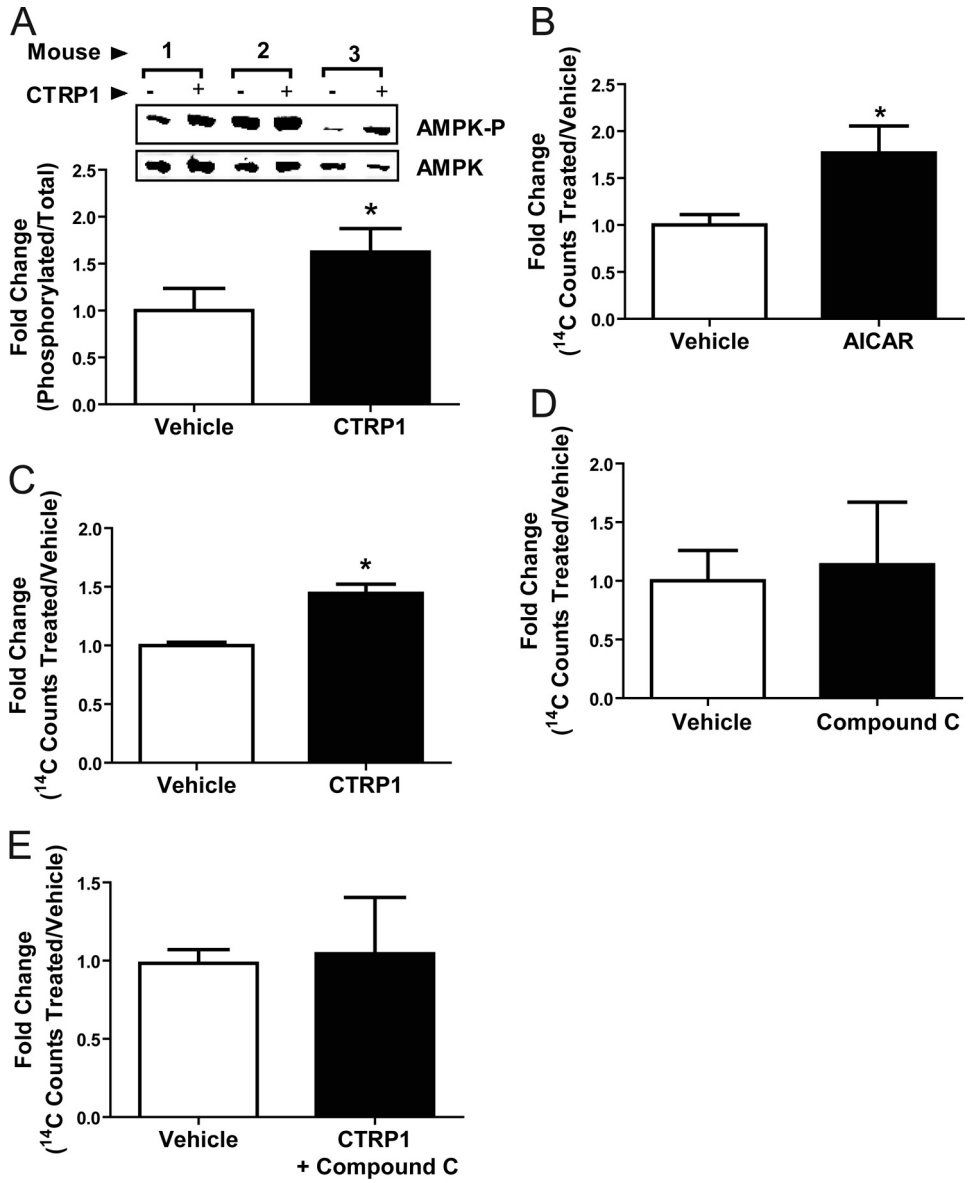

FIGURE 8. **Recombinant CTRP1 activates AMPK to increase fatty acid oxidation in isolated soleus muscle.** *A*, quantitative immunoblot analysis of AMPKα (Thr-172) phosphorylation (*AMPK-P*) in isolated soleus muscle ($n = 3$) stimulated with vehicle (−) or recombinant CTRP1 (+; 5 μg/ml). Phosphorylated protein levels were normalized to total protein levels. *B–E*, *ex vivo* fatty acid oxidation measurements in isolated soleus muscles ($n = 4$) treated with vehicle when compared with contralateral soleus muscles treated with AICAR (*B*, 2 mM), recombinant CTRP1 (*C*, 5 μg/ml), Compound C (*D*, 10 μM), or (CTRP1 + Compound C) (*E*), respectively. All values are reported relative to contralateral soleus muscle treated with vehicle controls. Values are mean -fold change ± S.E. *, $p < 0.05$ *versus* vehicle.

enzyme genes (*e.g. Acox1*, *Cpt1*, *Lcad*, *Mcad*) involved in fatty acid oxidation in skeletal muscle of Tg mice (data not shown). The well known adipokine adiponectin has also been shown to increase muscle fat oxidation (35, 39, 40). However, in CTRP1 Tg mice on an HF diet, circulating adiponectin levels were significantly lower (by ~30%) when compared with WT controls. Therefore, the observed increase in fat oxidation in Tg mice is due to the direct effect of overexpressing CTRP1 rather than an indirect effect resulting from changes in circulating levels of adiponectin.

Adiponectin is a multifunctional adipokine with pleiotropic effects on metabolic and inflammatory processes (1, 41). In adiponectin-null mice, we observed an ~2-fold increase in circulating levels of CTRP1 (7). Despite an ~30% reduction in circulating adiponectin levels, CTRP1 Tg mice are modestly more insulin-sensitive and gain less fat mass relative to littermate controls when challenged with an HF diet. Combined, these observations suggest possible partial functional compensation by CTRP1 in the absence of adiponectin. Indeed, our data provide an additional explanation, besides enhanced leptin sensitivity (20), for the relatively mild metabolic phenotypes seen in the adiponectin knock-out mice (16–19). It is not surprising that other secreted proteins may share overlapping function with adiponectin. In fact, adiponectin belongs to the C1q protein family that comprises over 30 conserved members in humans (11). Our recent studies suggest that CTRP3 (8), CTRP9 (6), and CTRP13 (9) also play important roles in regulating glucose and fatty acid metabolism.

Every CTRP has a unique tissue expression pattern (6, 7, 9, 42). In contrast to CTRP1, liver is the primary target tissue of CTRP3 *in vivo* (8). In mice, recombinant CTRP3 administration lowers blood glucose by suppressing hepatic gluconeogenic gene expression via activation of the protein kinase B/Akt signaling. In CTRP1 Tg mice, we did not observe any changes in hepatic gluconeogenic gene (*G6Pase* and *PEPCK*) expression (data not shown). Similarly, recombinant CTRP1 did not alter AMPK signaling, glucose output, gluconeogenic gene expression, or fatty acid oxidation in cultured rat H4IIE hepatocytes (data not shown). Administration of recombinant CTRP1 acutely induced AMPKα phosphorylation in skeletal muscle of wild-type mice. However, similar changes in AMPKα phosphorylation were not observed in CTRP3-treated myotubes (8) or skeletal muscles of mice injected with recombinant CTRP3.[5] Consistent with the acute effects of recombinant CTRP1 injection, we also observed chronic hyperphosphorylation of AMPKα and its downstream target, ACC, in skeletal muscle, but not liver, of Tg mice. Importantly, we showed that recombinant CTRP1 directly induces AMPKα phosphorylation and enhances fatty acid oxidation in isolated soleus muscle *ex vivo*. These results indicate that skeletal muscle is a target tissue of CTRP1 *in vivo*.

Leptin and adiponectin act in peripheral tissues as well as in the CNS. The central importance of leptin action in the hypothalamus to modulate food intake is unequivocal. Mutations in leptin or its receptor result in morbid obesity in humans and mice (43–45). In contrast, the role of adiponectin in the CNS is less clear. Although one study demonstrated that adiponectin acts centrally to reduce body weight by enhancing peripheral energy expenditure without affecting food intake (46), another concluded that adiponectin acts in the CNS to increase food intake and decrease energy expenditure (47). Additionally, it remains unclear how the multimeric forms of adiponectin cross the blood-brain barrier to gain access to the CNS (48). In wild-type mice, CTRP1 mRNA is expressed at relatively low levels in the brain (7), whereas in Tg mice, CTRP1 transgene expression is elevated in the brain. Despite increased expression of CTRP1 in the CNS, we observed no changes in food intake. Given the complex neurocircuitry governing ingestive behavior, it remains to be determined whether CTRP1 acts in a specific region of the brain to modulate food intake. Future studies are needed to address whether circulating CTRP1 can cross the blood-brain barrier under normal physiological conditions and whether CTRP1 may act in the CNS to regulate other physiological processes.

Three adiponectin receptors have been identified: AdipoR1, AdipoR2 (49), and T-cadherin (50). AdipoR1 and AdipoR2 are unusual seven-transmembrane proteins, having an inverse topology (*i.e.* N terminus facing the cytoplasm) when compared with classical G-protein-coupled receptors (GPCRs). In contrast, T-cadherin belongs to the cadherin family and lacks an intracellular tail; it is anchored to the plasma membrane via a glycophosphatidylinositol moiety. A fourth receptor expressed by macrophages has recently been suggested (51); the nature of this receptor is not known. Using knock-out mice, AdipoR1, AdipoR2, and T-cadherin have been shown to mediate the protective biological effects of adiponectin in various metabolic and cardiovascular disease models (52, 53), as well as in cancer (54). The proximal signaling events downstream of these receptors remain unresolved. How glycophosphatidylinositol-anchored T-cadherin couples adiponectin binding to the activation of signal transduction pathways (*e.g.* AMPK) in cells remains unclear. AdipoR1, AdipoR2, and T-cadherin are unlikely to act as receptors for CTRP1. Overexpressing these receptors in mammalian cells did not lead to enhanced binding of recombinant CTRP1, suggesting that CTRP1 binds to its own unique receptor.[6] AdipoR1 and AdipoR2 belong to the progestin and adipoQ receptor (PAQR) family of conserved seven-transmembrane proteins currently comprising 11 members encoded by distinct genes in humans (55). It remains to be determined whether any of the PAQR family members can serve as receptor for CTRP1.

In sum, we provided evidence that CTRP1 is a novel metabolic regulator that activates AMPK to control fatty acid metabolism. Future loss-of-function studies using CTRP1 knock-out mice will likely yield further insights into the mechanisms of action of CTRP1 in normal and disease states.

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