Mechanisms by which adiponectin reverses high fat diet-induced insulin resistance in mice

Xiruo Li a,b, Dongyan Zhang a, Daniel F. Vatners a , Leigh Goedeke a , Sandro M. Hira b a,c , Ye Zhang a,d, Rachel J. Perry a,b and Gerald I. Shulman a,b,1

a Department of Internal Medicine, Yale School of Medicine, New Haven, CT 06520; b Department of Cellular and Molecular Physiology, Yale School of Medicine, New Haven, CT 06520; c Institute of Physical Activity Sciences and Sports, Cruzeiro do Sul University, 03342 Sao Paulo, Brazil; and d Department of Endocrinology & Metabolism, First Hospital of Jilin University, 130021 Changchun, Jilin, China

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Adiponectin has emerged as a potential therapy for type 2 diabetes mellitus, but the molecular mechanism by which adiponectin reverses insulin resistance remains unclear. Two weeks of globular adiponectin (gAcrp30) treatment reduced fasting plasma glucose, triglyceride (TAG), and insulin concentrations and reversed whole-body insulin resistance, which could be attributed to both improved insulin-mediated suppression of endogenous glucose production and increased insulin-stimulated glucose uptake in muscle and adipose tissues. These improvements in liver and muscle sensitivity were associated with ~50% reductions in liver and muscle TAG and plasma membrane (PM)-associated diacylglycerol (DAG) content and occurred independent of reductions in total ceramide content. Reductions of PM DAG content in liver and skeletal muscle were associated with reduced PKCζ translocation in liver and reduced PKCβ and PKCζ translocation in skeletal muscle resulting in increased insulin-stimulated insulin receptor tyrosine1162 phosphorylation, IRS-1/IRS-2-associated PI3-kinase activity, and Akt-serine phosphorylation. Both gAcrp30 and full-length adiponectin (Acrgp30) treatment increased eNOS/AMPK activation in muscle and muscle fatty acid oxidation. gAcrp30 and Acrgp30 infusions also increased TAG uptake in epididymal white adipose tissue (eWAT), which could be attributed to increased lipoprotein lipase (LPL) activity. These data suggest that adiponectin and adiponectin-related molecules reverser lipoid-induced liver and muscle insulin resistance by reducing ectopic lipid storage in these organs, resulting in decreased plasma membrane sn-1,2-DAG–induced nPKC activity and increased insulin signaling. Adiponectin mediates these effects by both promoting the storage of TAG in eWAT likely through stimulation of LPL as well as by stimulation of AMPK in muscle resulting in increased muscle fat oxidation.

significance

As it is estimated that one in three Americans will suffer from type 2 diabetes by 2050, interventions to ameliorate insulin resistance are of great interest. Adiponectin has emerged as a promising insulin-sensitizing adipokine; however, the mechanisms by which adiponectin administration improves insulin sensitivity are unclear. Here, we show that globular adiponectin (gAcrp30) and full-length adiponectin (Acrgp30) reverse insulin resistance in HFD-fed mice through reductions in ectopic lipid in liver and muscle likely by stimulation of LPL activity in eWAT and increased eNOS/AMPK activation and fat oxidation in muscle. These effects, in turn, lead to decreased plasma membrane diacylglycerol content, resulting in decreased PKCζ activation in liver and decreased PKCβ/PKCζ activity in muscle and improved insulin signaling in these tissues.

type 2 diabetes mellitus (T2DM) is one of the leading causes of morbidity and mortality in the adult population worldwide (1, 2) and is associated with disease in many organ systems, including nonalcoholic fatty liver disease (NAFLD) and atherosclerotic vascular disease (ASCVD) (3–6). Insulin resistance plays a critical role in the pathogenesis of T2DM and the metabolic syndrome. The adipokine adiponectin has emerged as a potential antiadipotic, antiinflammatory, and antiatherogenic factor (7, 8). Unlike adipokines such as leptin, plasma adiponectin levels are inversely correlated with adiposity and decreased in obesity, insulin resistance, and T2DM (9, 10). Adiponectin is present in human plasma as full-length adiponectin (Acrp30) and as a C-terminal globular fragment (gAcrp30) (11–13). The C-terminal globular fragment is produced by proteolytic cleavage and is thought to be the pharmacologically active moiety (11). A wide variety of explanations for adiponectin’s glucose lowering and insulin sensitizing properties has been proposed, which have been derived predominantly from in vitro and ex vivo studies, including: suppression of gluconeogenesis (14–16), increased AMPK/ACC-dependent fatty acid oxidation in liver and muscle (7, 12, 14, 17), and reduced hepatic ceramide content by activation of hepatic ceramidase (18). A clear, consistent model for adiponectin’s action in vivo is lacking, and the mechanisms by which adiponectin ameliorates insulin resistance are a matter of active debate.

The association between ectopic lipid and insulin resistance in liver and skeletal muscle is widely recognized (19–21). Diacylglycerols (DAGs) and ceramides are the two best-studied mediators of lipid-induced insulin resistance. Ceramides have been shown to impair insulin action at the level of protein kinase B (Akt) phosphorylation, through activation of protein kinase Cζ (PKCζ) and/or protein phosphatase 2A (22–24). In contrast, plasma membrane sn-1,2-DAGs, which has been shown to be the key DAG stereoisomer, impair insulin action via activation of novel PKCs (nPKCs), including PKCζ in liver (25–27) and both PKCδ and PKCζ in skeletal muscle (28, 29). PKCζ activation subsequently impairs insulin receptor kinase (IRK) tyrosine kinase activity, and PKCζ activation impairs insulin signaling at the level of IRS-1/IRS-2–associated PI3-kinase activity (20, 30, 31). Insulin resistance in the liver leads to reduced insulin-stimulated hepatic glycogen synthesis and defects in insulin suppression of hepatic glucose production, while insulin resistance in the skeletal muscle leads to reduced insulin-stimulated muscle glucose transport. In the setting of white adipose tissue (WAT) insulin...
resistance, WAT lipolysis is resistant to suppression by insulin, leading to increased nonesterified fatty acid (NEFA) delivery to the liver and muscle, which may further promote increased liver and muscle ectopic lipid content (4, 5, 32, 33).

Given that prior studies have demonstrated that increased plasma adiponectin concentrations lead to accretion of WAT and improved glycemia in mice (34, 35), we hypothesized that the insulin-sensitizing properties of adiponectin might be due to protection against ectopic lipid deposition in insulin-responsive tissues. To address this hypothesis, we performed a comprehensive series of studies to assess the effects of 2 wk gAcrp30 and Acrp30 treatment on multiple metabolic fluxes using a combination of stable- and radio-labeled isotopic tracers, in a high fat diet (HFD)-fed mouse model of lipid-induced insulin resistance. Here, we demonstrate that 2 wk of gAcrp30 treatment reverses whole-body insulin resistance in HFD-fed mice by reducing plasma membrane DAG content, resulting in decreased translocation of PKCs to the plasma membrane in liver and decreased PKCθ/PKCθ translocation in skeletal muscle, leading to increased insulin signaling in both of these tissues. This reduction in ectopic lipid storage in liver and muscle could be attributed to increased lipoprotein lipase activity in epididymal WAT (eWAT), resulting in increased lipid uptake in eWAT, as well as activation of AMPK in muscle, which, in turn, promoted increased fatty acid oxidation in skeletal muscle. Taken together these results provide insights into the mechanisms by which adiponectin reverses insulin resistance in vivo.

Results

Two-Week Global Adiponectin Treatment Ameliorates Lipid-Induced Insulin Resistance. In order to examine the effect of long-term exposure to increased global adiponectin (gAcrp30) on glucose metabolism, we performed continuous subcutaneous (s.c.) gAcrp30 infusions (2.5 μg/d) in 12-wk HFD-fed mice for 2 wk. As expected, plasma adiponectin concentrations increased in the gAcrp30-treated mice compared with control mice (Fig. 1 A). To assess the effect of gAcrp30 on energy balance, metabolic cages were utilized and whole-body energy expenditure was determined by indirect calorimetry. Consistent with the lack of difference in body weight or body composition (SI Appendix, Fig. S1 A and B), we observed no effect of gAcrp30 on whole-body oxygen consumption, carbon dioxide production, energy expenditure, caloric intake, respiratory exchange ratio, drinking, or activity (SI Appendix, Fig. S1 C–D).

While gAcrp30 did not alter whole-body energy metabolism, plasma triglyceride (TAG) concentrations as well as liver TAG content and muscle TAG content were significantly reduced by 35%, 45%, and 60%, respectively (Fig. 1 B–D). Consistent with a reduction in ectopic lipid content in liver and skeletal muscle, mice treated for 2 wk with gAcrp30 exhibited a 10% reduction in plasma glucose concentrations and a 65% reduction in plasma insulin concentrations after overnight fasting (Fig. 1 E and F). In contrast, there was no difference in fasting plasma NEFA concentration between groups (SI Appendix, Fig. S2A). In order to determine the effects of gAcrp30 on tissue-specific insulin action, we performed hyperinsulinemic-euglycemic clamps combined with radiolabeled and stable isotopes. Basal endogenous glucose production (EGP) was reduced by 13% in the gAcrp30 group as compared with the control group (Fig. 1 G), resulting in reduced fasting plasma glucose concentrations (Fig. 1E). During the hyperinsulinemic phase of the clamp study, gAcrp30-treated mice displayed a twofold increase in the glucose infusion rate required to maintain euglycemia, reflecting increased whole-body insulin sensitivity (Fig. 1H and SI Appendix, Fig. S2 B and C). The increased whole-body insulin sensitivity could be attributed to both a twofold increase in insulin-mediated suppression of hepatic glucose production and a 15% increase in insulin-stimulated peripheral glucose disposal (Fig. 1 G and J and SI Appendix, Fig. S2D). Specifically, our data demonstrated that glucose uptake is increased by 50–100% in all assessed tissues, including skeletal muscle, WAT, and brown adipose tissue (Fig. 1 J–L).

Globular Adiponectin Reduces Plasma Membrane DAG Content and PKC Activation in Liver and Skeletal Muscle. As 2 wk of gAcrp30 treatment resulted in a marked improvement in liver and muscle insulin sensitivity, we next assessed insulin signaling pathways in the liver and skeletal muscle of these mice. Consistent with increased whole-body insulin sensitivity, gAcrp30-treated mice manifested twofold to fourfold increases in insulin-mediated insulin receptor tyrosine autophosphorylation (tyrosine 1162) in both liver and skeletal muscle (Fig. 2 A and B). We also observed fourfold increases in insulin-stimulated insulin receptor substrate-2 (IRS-2)–associated phosphoinositide 3-kinase (PI3K) activity in liver and IRS-1–associated PI3K activity in muscle, as well as twofold increases in Akt2 phosphorylation in liver and skeletal muscle of gAcrp30-treated mice as compared with vehicle-treated mice in the clamp state (Fig. 2 C–F), indicating improved insulin signaling in liver and muscle. Activated c-Jun N-terminal kinase (JNK) can phosphorylate insulin receptor substrate-1 (IRS-1) serine 302, resulting in negative regulation of the insulin signaling pathway in mouse tissues (7, 36). This mechanism may play a role in the improved insulin sensitivity seen in gAcrp30-treated mice, as we observed an ~40% decrease in JNK phosphorylation in liver and muscle from animals treated with gAcrp30 vs. vehicle-treated animals (SI Appendix, Fig. S2 E and F), which may in part be due to adiponectin’s effect on reducing oxidative stress (7).

DAGs and ceramides are two well-studied bioactive lipids that have been proposed to mediate lipid-induced insulin resistance (27). Plasma membrane DAGs have been shown to mediate insulin resistance by activation of nPKCs, specifically PKCs in the liver and both PKCs and PKCθ in the skeletal muscle (25, 30, 37, 38). Among the three stereoisomers of DAG (SN-1,2-DAG, SN-1,3-DAG, and SN-2,3-DAG), SN-1,2-DAG is thought to be primarily responsible for nPKC activation (39–41). To understand the mechanism by which gAcrp30 treatment ameliorates lipid-induced liver and muscle insulin resistance, DAG content, ceramide content, and nPKC translocation were measured in these tissues. Hepatic plasma membrane SN-1,2-DAG was decreased by 35% in gAcrp30-treated mice, which was associated with a ∼50% reduction in PKCs membrane translocation, reflecting reduced PKCθ activation (Fig. 2 G and H). Plasma membrane SN-2,3-DAG content was decreased by 35% without any difference in SN-1,3-DAG content and SN-1,2-DAG content in other subcellular compartments (SI Appendix, Fig. S2 G–J). INSIR Thr1160 is a PKCθ target, upon which phosphorylation impairs the tyrosine kinase activity of the insulin receptor and, thereby, diminishes downstream insulin signaling (25, 40). Consistent with reductions in PKCθ activity and improved hepatic insulin sensitivity, hepatic insulin receptor Thr1160 phosphorylation was decreased in gAcrp30-treated mice (Fig. 2 I). Similarly, in the gastrocnemius muscle, gAcrp30-treated mice exhibited an ~55% reduction in plasma membrane DAG content with an associated 60–80% reduction in PKCθ and PKCζ translocation (Fig. 2 J–L). In contrast, despite the reductions in liver and muscle TAG content, plasma membrane DAG content, and marked reversal of insulin resistance in liver and skeletal muscle, there were no significant changes in total ceramide content in these tissues (Fig. 2 M and N), arguing against an important role for adiponectin-induced activation of ceramidase as the insulin-sensitizing mechanism by which adiponectin would have been expected to lead to a reduction in total ceramide content (18). In addition, we did not observe any significant differences in the total content of specific ceramide species (C16:0 and C18:0), which have been specifically hypothesized to mediate insulin resistance in rodents (42, 43) (SI Appendix, Fig. S2 J–M). While gAcrp30 treatment did not cause a reduction in total tissue ceramide content, it did result in reductions in several hepatic ceramide species (C16:0, C20:0, C22:0, C24:0, and C24:1) in the
plasma membrane (SI Appendix, Fig. S2N), which correlated with the improved insulin sensitivity in liver.

**gAcrp30 Improves Insulin Signaling in WAT.** Next, we sought to understand the effect of gAcrp30 treatment on insulin signaling in WAT and on WAT lipolysis. gAcrp30 administration increased phosphorylation of IRK and Akt2, and reduced phosphorylation of perilipin, adipose TAG lipase (ATGL), and hormone-sensitive lipase (HSL) in the clamp state, indicating improved insulin signaling in WAT (Fig. 3A and B and SI Appendix, Fig. S3 A–C). Consistent with these data, gAcrp30-treated mice had reduced whole-body glycerol turnover rate in the basal and clamp state, demonstrating that gAcrp30 treatment reduced WAT lipolysis and improved insulin signaling in WAT (Fig. 3C). Reduced glycerol conversion to glucose may result in reduced hepatic glucose production and plasma glucose concentrations (44). However, surprisingly, there were no differences in the whole-body fatty acid turnover rate or plasma NEFA concentrations (SI Appendix, Fig. S3 D and E), suggesting that gAcrp30 may also promote WAT reesterification. Consistent with
Fig. 2. Globular adiponectin reduces membrane DAG content and nPKC activation in liver and muscle. (A and B) Western blot images for insulin receptor kinase phosphorylation (pY1162) in liver (n = 5–7) and skeletal muscle (n = 5) of control and gAcrp30-treated mice under the hyperinsulinemic-euglycemic clamp condition. Quantification is shown below. (C) IRS-2–associated PI3K activity in liver. (D) IRS-1–associated PI3K activity in muscle (n = 5). (E and F) Western blot images for Akt phosphorylation (pS473) in liver (n = 5–7) and skeletal muscle (n = 5) in the clamp state. Quantification is shown below. (G) Hepatic plasma membrane sn-1,2-DAG content. (H) Hepatic membrane/cytosolic PKCe ratio. Quantification is shown below. (I) Western blot images for insulin receptor kinase phosphorylation (pY1160) in liver (n = 5). Quantification is shown below. (J) Membrane DAG content in skeletal muscle. (K and L) Membrane/cytosolic PKCθ and PKCe ratio in skeletal muscle. PKCθ and PKCe were probed from the same membrane and therefore have the same corresponding loading controls (GAPDH and NaK-ATPase). Quantification is shown below. (M and N) Total ceramide content in liver (n = 16) and skeletal muscle. Data are shown as mean ± SEM *P < 0.05, **P < 0.01, ***P < 0.001 by unpaired Student’s t test.
the lack of differences in fatty acid turnover, we observed no differences in hepatic acetyl-CoA, malonyl-CoA, or long-chain acyl-CoA concentrations (SI Appendix, Fig. S3 F–H). Taken together, these data indicate that gAcrp30 treatment also improves insulin signaling in WAT and may affect WAT lipolysis and reesterification.

Globular Adiponectin Treatment Promotes a Switch from Glucose to Fat Oxidation in Skeletal Muscle. In order to determine whether the reduction in ectopic lipid (TAG/DAG) content could be attributed to increased fatty acid oxidation in liver and muscle, we assessed mitochondrial function in vivo and ex vivo. We employed positional isotopomer NMR tracer analysis (PINTA) to assess the effects of gArcp30 on in vivo hepatic citrate synthase flux (VCS, i.e., mitochondrial oxidation) and hepatic pyruvate carboxylase flux (VPC, i.e., gluconeogenesis from pyruvate) (45) and observed no significant differences in hepatic VPC or VCS in gAcrp30-treated mice (SI Appendix, Fig. S3 I and J). In addition, there was no difference in the phosphorylation of two key regulators of hepatic fatty acid oxidation and biosynthesis: 5′ AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC) with gAcrp30 treatment (SI Appendix, Fig. S3 K and L). In summary, no differences were observed in hepatic mitochondrial oxidation rate or its upstream regulators or downstream outflow (VPC) in the gAcrp30-treated mice.

Relative rates of mitochondrial ketone oxidation and β-oxidation (VFAO) normalized to citrate synthase flux (VCS) were determined in vivo in multiple tissues. gAcrp30 treatment promoted a shift away from glucose to other substrates (fatty acids, ketones, ketogenic amino acids) in gastrocnemius muscle (Fig. 3D), despite no effect on liver or quadriceps muscles (SI Appendix, Fig. S3 M and N). To further examine the effects of gAcrp30 on absolute rates of fatty acid oxidation and glucose oxidation in muscle, we assessed rates of 14CO2 production in isolated soleus muscle with [1-14C]palmitic acid and [14C6]D-glucose as substrates. Consistent with the in vivo gastrocnemius data, both fatty acid oxidation and glucose oxidation were increased in the Acrp30-treated and gAcrp30-treated soleus muscles (Fig. 3E and SI Appendix, Fig. S3O). To understand the potential molecular mechanisms by which fatty acid oxidation was increased in the soleus muscle, we measured phosphorylation of AMPK, ACC, and endothelial nitric oxide synthase (eNOS). Previous studies have shown that there is a positive feedback loop between nitric oxide production and AMPK activation (46). Consistent with these studies, we observed significant increases in phosphorylation of AMPK, ACC, and eNOS in the skeletal muscle of both Acrp30-treated and gAcrp30-treated mice (Fig. 3 F–H).
These data suggest that gAcrp30 and Acrp30 treatment activates the eNOS/AMPK/ACC pathway and promotes a switch from glucose oxidation to fatty acid oxidation in predominately slow-twitch gastrocnemius and soleus muscles but does not impact hepatic mitochondrial fat oxidation.

Globular Adiponectin and Full-Length Adiponectin Increase Lipoprotein Lipase Activity and Lipid Uptake in eWAT. To determine whether adiponectin treatment alters ectopic lipid deposition by changing TAG-rich lipoprotein metabolism, we performed a series of studies assessing very-low-density lipoprotein (VLDL) production and chylomicron clearance. We first measured the rates of hepatic VLDL-TAG production to evaluate whether hepatic VLDL-TAG production contributed to the reduced plasma TAG in the gAcrp30-treated mice. No significant difference in the hepatic VLDL-TAG production rate with gAcrp30 treatment was observed (SI Appendix, Fig. S4 A and B). Then, we tested the hypothesis that the reductions in TAGs and membrane DAGs in liver and skeletal muscle may be explained by increased uptake of lipids into WAT, thereby diverting circulating TAGs away from storage in liver and skeletal muscle. Consistent with the hypothesis, plasma lipid clearance was increased during an oral lipid tolerance test in the gAcrp30-treated mice (Fig. 4 A and B). gAcrp30 treatment promoted increased lipid uptake in eWAT despite no significant difference in lipid uptake in s.c. WAT (sWAT) or skeletal muscle (Fig. 4 C and D and SI Appendix, Fig. S4C).

Lipoprotein lipase (LPL) plays an important role in the clearance of plasma TAG and the import of TAG-derived fatty acid to muscle and heart for utilization and adipose tissues for storage (47). We measured plasma and tissue-specific LPL activity to assess whether gAcrp30 alters adipose chylomicron clearance via alterations in LPL activity. gAcrp30-treated mice have increased heparin-releasable LPL activity in plasma and increased LPL activity in eWAT and heart (Fig. 4 E–G). In contrast, there were no significant effects of gAcrp30 treatment on sWAT, brown adipose tissue (BAT), or skeletal muscle LPL activity (Fig. 4H and SI Appendix, Fig. S4 D and E).

It has previously been shown that Acrp30 also reduces plasma and tissue TAG content in mice liver and skeletal muscle (48, 49).

**Fig. 4.** Globular adiponectin and full-length adiponectin increase lipoprotein lipase activity and lipid uptake in epidydimal WAT. (A) Plasma TAG concentrations of control and gAcrp30-treated mice during oral lipid tolerance test. (B) Area under the plasma TAGs curve of control and gAcrp30-treated mice. (C and D) TAG uptake in epidydimal WAT (n = 8–10) and s.c. WAT of control and gAcrp30-treated mice. (E) Postheparin plasma LPL activity of control and gAcrp30-treated mice (n = 5). (F) eWAT LPL activity of control and gAcrp30-treated mice (n = 5). (G) Heart LPL activity of control and gAcrp30-treated mice. (H) s.c. white adipose LPL activity of control and gAcrp30-treated mice (n = 5). (I) Plasma TAG concentrations of control and Acrp30-treated mice during oral lipid tolerance test. (J) Area under the plasma TAGs curve of control and Acrp30-treated mice. (K and L) TAG uptake in eWAT and sWAT of control and Acrp30-treated mice. (M) Postheparin plasma LPL activity of control and Acrp30-treated mice. (N) eWAT LPL activity of control and Acrp30-treated mice. (O) Brown adipose tissue LPL activity of control and Acrp30-treated mice. (P) s.c. white adipose LPL activity of control and Acrp30-treated mice. Data are shown as mean ± SEM *P < 0.05, ***P < 0.001 by unpaired Student’s t test.
49). To determine whether the decreased TAG content by Acrp30 could be explained by similar mechanisms as gAcrp30 treatment, we performed continuous s.c. Acrp30 infusions (10 μg/d) in HFD-fed mice for 2 wk and a series of studies assessing chylomicron clearance and LPL activity. As expected, plasma TAG was decreased with Acrp30 treatment (Fig. 4I). Analogous to what we observed in gAcrp30 treated mice, 2-wk Acrp30 treatment increased lipid clearance during the oral lipid tolerance test and improved lipid uptake in eWAT, without significant difference in lipid uptake in sWAT or skeletal muscle (Fig. 4 J–L, and SI Appendix, Fig. S4F). Acrp30 infusion also increased heparin-releasable plasma LPL activity and increased LPL activity in eWAT and BAT (Fig. 4 M–O). No significant differences in sWAT, heart, and muscle were observed (Fig. 4P and SI Appendix, Fig. S4 G and H). Taken together, these data demonstrate that both full-length and globular adiponectin treatment enhances lipid uptake in eWAT, which may be attributed to localized stimulation of LPL activity in eWAT.

Discussion

WAT is not only a critical energy storage depot, but it also acts as an endocrine organ sensing metabolic signals and secreting hormones and adipocytokines (e.g., leptin and adiponectin) that regulate whole-body energy homeostasis (50–53). Consistent with previous reports (14, 15, 17), we have demonstrated that administration of globular adiponectin results in an improvement in whole-body glucose homeostasis. Despite great interest in adiponectin, previous reports (14, 15, 17), we have demonstrated that administration of globular adiponectin treatment enhances lipid uptake in eWAT, which may be attributed to localized stimulation of LPL activity in eWAT.

Adiponectin receptor associated ceramidease activity, promoting decreased total hepatic ceramide content and ceramide-induced insulin resistance, has been proposed to mediate adiponectin’s insulin-sensitizing properties (18). However, in contrast to this hypothesis, we dissociated changes in total ceramide content in the liver and skeletal muscle from gAcrp30-induced improvements in liver and muscle insulin sensitivity. We also did not observe any significant differences in the content of specific ceramide species (C16:0 and C18:0), which have been specifically hypothesized to mediate insulin resistance in rodents (42, 43). While gAcrp30 treatment did not cause a reduction in total tissue ceramide content or in changes in C16:0 or C18:0 ceramides, it did result in reductions in several hepatic ceramide species (C16:0, C20:0, C22:0, C24:0, and C24:1) in the plasma membrane, which correlated with improved insulin sensitivity in liver. Whether these specific plasma membrane-associated ceramide species also contributed to alterations in insulin action will need to be examined in future studies.

Nevertheless, ceramide-induced insulin resistance is thought to alter downstream insulin signaling at the level of Akt; however, we observed that gAcrp30 improved insulin action at the level of the insulin receptor, which is not compatible with the putative mechanisms by which adiponectin is thought to mediate insulin resistance at the level of AKT2 phosphorylation.

In contrast with ceramide-induced insulin resistance, DAG-PKCε–induced insulin resistance can explain improved insulin signaling at the level of the insulin receptor. By this mechanism, sn-1,2-DAG accumulation in the plasma membrane of liver and muscle results in nPKC translocation from the cytoplasm to the plasma membrane, leading to decreased insulin signaling at the level of the insulin receptor due to PKCε activation and at the level of IRS-1–associated and IRS-2–associated PI3-kinase due to PKCθ activation (25, 26, 30). We observed that 2 wk of gAcrp30 treatment reduced plasma membrane sn-1,2-DAG in liver and membrane-associated DAG in muscle, leading to decreased PKCε:activity in liver and both PKCθ and PKCε:activity in skeletal muscle. As a result, insulin signaling at the level of insulin receptor kinase increased in both of these tissues. As such, the effect of global adiponectin on tissue-specific insulin action appears to occur through reductions in skeletal muscle plasma membrane DAG content, resulting in reduced PKCε:activation in liver and reduction in both PKCε: and PKCθ: activation in skeletal muscle.

In both in vitro and ex vivo studies, adiponectin has been suggested to reduce TAG content in the liver and muscle by enhancing fatty acid oxidation in an AMPK-dependent manner (7, 12, 14, 54, 55). However, Yamauchi et al. found that globular adiponectin cannot activate hepatic AMPK signaling pathways (48). No competing hypothesis has yet been published, and so the underlying physiological mechanisms by which gAcrp30 reduces hepatic TAG are still debated. Further complicating this question, most mechanistic studies examining adiponectin’s mechanism of action have been performed purely in vitro and ex vivo, whereas in vivo studies are critical to understand the complex interorgan cross-talk that regulates metabolic physiology. Reduced ectopic lipid content in liver and skeletal muscle may be due to several factors including 1) decreased NEFA flux to these tissues from reduced WAT lipolysis; 2) increased rates of tissue mitochondrial fatty acid oxidation; and 3) decreased lipid delivery to tissues from circulating lipoproteins. We evaluated each of these potential mechanisms for the gAcrp30-induced reductions in ectopic lipids in HFD-fed mice using a comprehensive series of in vivo metabolic studies. While gAcrp30 appeared to suppress rates of WAT lipolysis, as reflected by reduced rates of glycerol turnover and increased WAT insulin sensitivity, as reflected by increased insulin-stimulated glucose uptake, it did not affect whole-body fatty acid turnover potentially due to compensatory changes in reesterification. Additionally, hepatic mitochondrial fatty acid oxidation and the regulation of fat oxidation in liver were unchanged. In gastrocnemius and soleus muscle, gAcrp30 treatment increases muscle fatty oxidation in vivo and ex vivo, an effect that was correlated with increased phosphorylation of ACC in a manner consistent with previously described eNOS/AMPK-dependent regulation of ACC (46). Reduced content in liver and skeletal muscle DAG could account, in part, for the reduced ectopic lipid deposition seen in several tissues in gAcrp30-treated mice and the improvement in muscle insulin sensitivity.

In addition to promoting increased muscle fatty acid oxidation, we also found that both gAcrp30 and Acrp30 treatment reduces ectopic lipid (TAG/plasma membrane DAG) accumulation in liver and skeletal muscle by improving WAT TAG uptake and further increasing WAT storage capacity. Adiponectin treated mice displayed increased LPL activity in postheparin plasma and eWAT and improved adipose postprandial triacylglycerol uptake. These results are consistent with our observations that 2 wk of gAcrp30 or Acrp30 treatment increased eWAT mass but did not change total fat mass, as assessed by 1H NMR.

Our findings also imply an important role for decreased plasma adiponectin in the development of lipid-induced liver and skeletal muscle insulin resistance. In humans and monkeys, plasma adiponectin levels correlate significantly with whole-body insulin sensitivity (56, 57). Overexpression or administration of adiponectin in mice results in a decrease in hyperglycemia and improvement in systemic insulin sensitivity (7, 58), whereas adiponectin-deficient mice exhibit impaired insulin sensitivity and are prone to diabetes (8, 59). Tying all of this together, circulating adiponectin may be a reflection of the presence of functioning adipose tissue, a part of the machinery the WAT uses in its fat-storing operation. In normal physiology, healthy
Materials and Methods

Adipose tissue secretes sufficient adiponectin to promote storage of circulating TAG in WAT and signal a shift to increase fatty acid oxidation in skeletal muscle. However, in obesity, as adipose tissue has limited storage capacity, WAT secretion of adiponectin decreases. This derangement in fat storage and muscle fat oxidation may then lead to increased ectopic lipid (TAGs/plasma membrane DAGs) accumulation in liver and skeletal muscle and the subsequent development of insulin resistance in these organs leading to the metabolic syndrome, hepatic steatosis/NASH, and atherosclerosis.

Taken together, these results suggest that chronic adiponectin administration ameliorates insulin resistance in an HFD-fed mouse model of obesity, NAFLD, and insulin resistance by two major mechanisms. First, adiponectin treatment promotes increased WAT LPL activity, which may lead to increased uptake of TAG into WAT, thus diverting circulating TAG away from storage in liver and skeletal muscle. Second, adiponectin treatment promotes increased fatty acid oxidation in skeletal muscle, which, in turn, may be attributed to the activation of AMPK and eNOS. These two effects of adiponectin, in turn, lead to reductions in liver and muscle plasma membrane-associated sn-1,2-DAG content, resulting in decreased PKCθ activity in liver and decreased PKCε and PKCθ in muscle resulting in increased insulin signaling and insulin action in these tissues. Furthermore, adiponectin-induced improvement in liver and muscle insulin sensitivity in insulin-resistant, HFD-fed mice occurred independently of changes in total ceramide content in these tissues. Together, these studies provide insights into the mechanisms by which adiponectin reverses HFD-induced liver and muscle insulin resistance in mice.

Flux Measurement. Positional isotope NMR tracer analysis (PINTA) was applied to measure rates of hepatic mitochondrial citrate synthase flux (VCS) and pyruvate carboxylase flux (VPC) as previously described (45). Infusion of [3-3H]glucose (PerkinElmer) at a rate of 0.05 μCi/min and [3,13C]sodium lactate (Cambridge Isotopes) at a rate of 40 μmol/kg/min was performed for a total of 120 min to measure VPC/VCS and VPC/VPEG as we previously described (45).

The ratio of pyruvate dehydrogenase flux to citrate synthase flux (VPC/VCS) was used to indicate tissue-specific metabolic substrate oxidation after a 2-h infusion of [1,2,3,4,5,6-13C6]glucose (16.7 μmol/kg min) for 5 min, 5.6 μmol/kg/min (continuous infusion) as previously described (61). Briefly, VPC/VCS was measured as the ratio of [4,5,13C]glutamate/[13C]alanine, [13C]alanine enrichment was measured by gas chromatography–mass spectrometry (GC/MS) and [4,5,13C]glutamate enrichment was measured by liquid chromatography–tandem mass spectrometry (LC-MS/MS) as previously described (29).

Materials and Methods

Animals. All rodent studies were approved by the Yale University Institutional Animal Care and Use Committee. Male C57BL/6J mice (Jackson Laboratory), were group housed at the animal care facility at Yale University Animal Research Center and maintained under controlled temperature (23 °C) and lighting (12:12 h light/dark cycle, lights on at 7:00 A.M.) with free access to water and food. Diet-induced obesity studies were carried out by feeding mice a HFD (60% calories from fat, Research Diets D12492). To study the effects of adiponectin treatment, following 2 wk or 10 wk of HFD, mini-osmotic pumps (Alzet) containing recombinant mouse globular adiponectin protein (Abcam), recombinant mouse full-length adiponectin (Abcam), or vehicle (saline) were implanted s.c. Adiponectin was released at a rate of 2.5 μg/d (globular adiponectin) or 10 μg/d (full-length adiponectin) for 14 d based on previous literature (14, 48). Food and water intake measurements and body weight and adiposity were performed in a Columbus Lab Animal Monitoring System metabolic cages (Columbus Instruments). During this time, food intake and body weight were regularly monitored. The mice used for euglycemic clamp and in vivo tracer studies underwent surgery under isoflurane anesthesia to place catheters in the jugular vein and single-housed mice were allowed to recover 6–7 d before planned experiments.

Hyperinsulinemic-Euglycemic Clamps. Clamps were performed as previously described (26, 60). Briefly, after an overnight fast, a 120-min basal infusion with [3-3H]glucose (PerkinElmer) at a rate of 0.05 μCi/min, [1,2,3,3-D3]-glycerol (Sigma Aldrich) at a rate of 1.5 μmol/kg min and potassium [13C6] palmitate (Cambridge Isotopes) at a rate of 0.7 μmol/kg min was performed. After the basal period, mice underwent a 140-min hyperinsulinemic-euglycemic clamp by infusing [3-3H]glucose, [1,2,3,3-D3]-glycerol, and potassium [13C6] palmitate at the rates indicated above, and in the last 5 min of clamp period, insulin was infused at 2–3 μU/mg/min (DO-2-deoxy-[1,4-13C]glucose (DO-2-DG) PerkinElmer) was given to estimate tissue-specific glucose uptake. Twenty percent dextrose (Hospira) at a variable rate and insulin at a rate of 3 mU/kg min was infused through the jugular venous catheter to maintain a steady-state plasma glucose concentration of ~120 mg/dL. Plasma glucose concentrations were measured every 10–15 min during the hyperinsulinemic-euglycemic clamp period. At the end of the study, mice were euthanized with intravenous (i.v.) pentobarbital and tissues were dissected and placed in tubes precooled with liquid nitrogen. The specific activity of glucose was measured in plasma samples collected at the steady state during basal and clamp by liquid scintillation counting.
IRS-2–associated PI3Kα activities were measured in liver and muscle extracts after immunoprecipitation with IRS-1 antibody (BD Transduction Laboratories) or IRS-2 antibody (Cell Signalling Technology). Membranes were incubated overnight at 4°C. Then, the incorporation of ³²P into PI to yield phosphatidylinositol-3-monophosphate was measured as described (69, 70). Briefly, for plasma LPL activity, blood samples were collected after overnight fasting to determine basal plasma TAG and LPL activity. Then, mice were injected i.v. with heparin (50 μg/kg of body weight) and blood samples were taken after 10-min injection. Postheparin plasma LPL activity was assessed by a fluorometric assay (Cell Biologics). Tissue LPL was extracted by incubation of tissue at 37°C for 1 h in phosphate-buffered saline (PBS) with 5 μM heparin and 2 mg/mlBSA. Samples were centrifuged at 9000 × g for 15 min, and the supernatant tissue LPL activities were measured in the presence of heat-inactivated mouse serum using a fluorometric assay (Cell Biologs).

**Statistical Analysis.** All data are expressed as the mean ± SEM. Results were assessed using two-tailed unpaired Student’s t test or two-way ANOVA. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

GraphPad Prism 8.0 was used for all statistical analyses. In most cases, n = 6–9 per group, unless otherwise indicated in the figure legends.

**Data Availability.** All study data are included in the article and SI Appendix.

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