Targeted Induction of Ceramide Degradation Leads to Improved Systemic Metabolism and Reduced Hepatic Steatosis

Graphical Abstract

Highlights
- Ceramides play a causal role in diet-induced NAFLD
- Degradation of ceramides in liver results in improved glucose and lipid metabolism
- Degradation of ceramide in fat results in improved glucose and lipid metabolism
- Ceramide promotes PKCζ activation and CD36-mediated lipid uptake in the liver

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In Brief
Xia et al. reveal a “cross-talk” between the liver and adipose tissue using mice that inducibly express acid ceramidase, which triggers the deacylation of ceramides in these tissues. Decreased ceramide levels result in reduced activation of the ceramide-activated protein kinase C zeta, leading to reduced hepatic steatosis and improved insulin action.
Targeted Induction of Ceramide Degradation Leads to Improved Systemic Metabolism and Reduced Hepatic Steatosis

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SUMMARY

Sphingolipids have garnered attention for their role in insulin resistance and lipotoxic cell death. We have developed transgenic mice inducibly expressing acid ceramidase that display a reduction in ceramides in adult mouse tissues. Hepatic overexpression of acid ceramidase prevents hepatic steatosis and prompts improvements in insulin action in liver and adipose tissue upon exposure to high-fat diet. Conversely, overexpression of acid ceramidase within adipose tissue also prevents hepatic steatosis and systemic insulin resistance. Induction of ceramidase activity in either tissue promotes a lowering of hepatic ceramides and reduced activation of the ceramide-activated protein kinase C isoform PKCζ, though the induction of ceramidase activity in the adipocyte prompts more rapid resolution of hepatic steatosis than overexpression of the enzyme directly in the liver. Collectively, our observations suggest the existence of a rapidly acting “cross-talk” between liver and adipose tissue sphingolipids, critically regulating glucose metabolism and hepatic lipid uptake.

INTRODUCTION

Numerous studies in humans and animals have shown that hepatic steatosis is strongly associated with insulin resistance. Mice challenged with a high-fat diet (HFD) develop severe insulin resistance and hepatic steatosis (Birkenfeld and Shulman, 2014; Shimomura et al., 1999a, 1999b). However, the causal relationship between hepatic steatosis and insulin resistance is unclear and controversial. Murine models with altered hepatic lipid storage (Monetti et al., 2007; Yu et al., 2005), mobilization (Brown et al., 2010; Hoy et al., 2011; Minehira et al., 2008), and oxidation (Monsénégó et al., 2012) all exhibit greatly increased hepatic lipid accumulation without accompanying insulin resistance. Conversely, alterations in hepatic insulin signaling are sufficient to induce hepatic steatosis (Taniguchi et al., 2005).

Ceramides are important members of the sphingolipid family and are essential precursors for complex sphingolipids. A series of studies have shown that increased ceramide levels in both liver and plasma coincide with the development of liver dysfunction, hepatic insulin resistance, and steatosis in rodents (Ichi et al., 2007; Xia et al., 2014; Yetukuri et al., 2007). Previous work has identified the liver as a target of ceramide-induced insulin resistance and inhibition of whole-body ceramide synthesis reduces obesity-induced insulin resistance in rodents (Holland et al., 2007). In particular, ceramides derived from C16 fatty acids appear to oppose insulin action most potently (Raichur et al., 2014; Turpin et al., 2014). Breakdown of ceramides are initiated by enzymes called ceramidases, which are categorized by homology and pH optima at which they can hydrolyze ceramides into sphingosines and free fatty acids (Xia et al., 2014). The anti-diabetic and anti-steatotic adipokine, adiponectin, rapidly lowers hepatic ceramide content, thereby improving glucose homeostasis through its receptor-associated ceramidase activity (Holland et al., 2011). Similarly, overexpression of acid ceramidase in cultured cells prevents saturated fatty acids from impairing insulin action in cultured C2C12 myotubes (Chavez et al., 2003).

Previous studies on the role of sphingolipid biosynthetic enzymes in systemic metabolism have employed constitutive gain-of-function or loss-of-function models. However, the usefulness of these constitutive models has been limited by complex phenotypes due to compensatory mechanisms and developmental issues. To further investigate the physiological effects of an acute increase in ceramidase activity in a tissue-specific manner, we have developed transgenic mice that express acid ceramidase under the control of a tetracycline response element (TRE) and are essential precursors for complex sphingolipids. A series of studies have shown that increased ceramide levels in both liver and plasma coincide with the development of liver dysfunction, hepatic insulin resistance, and steatosis in rodents (Ichi et al., 2007; Xia et al., 2014; Yetukuri et al., 2007). Previous work has identified the liver as a target of ceramide-induced insulin resistance and inhibition of whole-body ceramide synthesis reduces obesity-induced insulin resistance in rodents (Holland et al., 2007). In particular, ceramides derived from C16 fatty acids appear to oppose insulin action most potently (Raichur et al., 2014; Turpin et al., 2014). Breakdown of ceramides are initiated by enzymes called ceramidases, which are categorized by homology and pH optima at which they can hydrolyze ceramides into sphingosines and free fatty acids (Xia et al., 2014). The anti-diabetic and anti-steatotic adipokine, adiponectin, rapidly lowers hepatic ceramide content, thereby improving glucose homeostasis through its receptor-associated ceramidase activity (Holland et al., 2011). Similarly, overexpression of acid ceramidase in cultured cells prevents saturated fatty acids from impairing insulin action in cultured C2C12 myotubes (Chavez et al., 2003).

Previous studies on the role of sphingolipid biosynthetic enzymes in systemic metabolism have employed constitutive gain-of-function or loss-of-function models. However, the usefulness of these constitutive models has been limited by complex phenotypes due to compensatory mechanisms and developmental issues. To further investigate the physiological effects of an acute increase in ceramidase activity in a tissue-specific manner, we have developed transgenic mice that express acid ceramidase under the control of a tetracycline response element cell type-specifically. This allows us to induce ceramide deacetylation in response to doxycycline exposure, resulting in the degradation of ceramides made within the cell or following uptake from serum. These models reveal profound improvements in hepatic steatosis and glucose metabolism with strong evidence for interorgan cross-talk as sphingolipids are shunted back and forth between liver and adipose. Thus, this system enables us to probe the impact of a genetically induced, acute...
change in ceramide levels on local and systemic insulin sensitivity.

RESULTS

Overexpression of Acid Ceramidase in the Liver Reduces Hepatic Ceramide Levels and Improves Hepatic and Adipose Insulin Sensitivity

Myriocin, an inhibitor of de novo ceramide synthesis, also robustly protects against hepatic steatosis when wild-type mice are maintained on a HFD (Figure S1A). To disrupt the over-accumulation of ceramides in the liver, we generated an inducible, liver-specific acid ceramidase transgenic (Alb-AC) mouse, which combines three transgenic lines: the albumin promoter-driven Cre line, a transgenic line carrying a Rosa26 promoter-driven loxP-stop-loxP-reverse tetracycline-controlled transactivator (rtTA) gene, and a Tet-responsive mouse acid ceramidase transgenic line (TRE-AC) (Figure S1B). In the absence of doxycycline, there is only a low level of endogenous acid ceramidase message expressed when using a primer set recognizing both transgenic and endogenous acid ceramidase sequences. Upon treatment with doxycycline, AC gene expression in the triple transgenics reaches approximately four times the levels seen in wild-type (WT) littermates. The overexpression is liver-specific, as it is not detected in any other tissues (Figure S1C). The increase in hepatic AC mRNA levels resulted in a 3-fold increase in acidermic acid enzyme activity in the liver (Figure S1D).

Serum ceramidase activity was not changed between WT and Alb-AC mice after doxycycline induction (Figure S1E).

We challenged the WT and Alb-AC mice with a HFD (60% calories from fat) containing 200 mg/kg doxycycline (HFD-dox). After 8 weeks of HFD-dox exposure, wild-type mice showed a 2.4-fold increase in hepatic ceramides compared to Chow-fed controls (Figure S1F). Alb-AC mice showed a significant reduction in C_{16:0} and C_{18:0} hepatic ceramide species (Figure 1A). In parallel, a significant lowering of C_{16:0} and C_{18:0} ceramide species was also observed in the serum (Figure 1A). Interestingly, although levels of liver sphingoid species did not change significantly compared to WT except for the levels of sphingosine, serum sphingoid species exhibited a sharp decline compared to WT (Figure S1G). Since past evidence showing the negative correlation between the signaling lipid diacylglycerol (DAG) and hepatic steatosis and insulin resistance (Shulman, 2014), we also measured hepatic levels of DAGs. Surprisingly, we found that levels of DAG in the liver were significantly higher in our Alb-AC mice compared to WT (Figure 1B). Transcriptionally, we found mRNA levels of DGAT1 decreased to 30% of WT, while levels of DGAT2 increased to ~5-fold above WT levels (Figure S1H). Serum levels of DAGs were comparable between WT and Alb-AC mice (Figure 1B).

We exposed Alb-AC and WT littermate control mice to HFD-dox for 8 weeks. We observed similar weight gain curves for the Alb-AC and WT mice and comparable body weights at the end of the 8 weeks of HFD-dox exposure (Figure S1I). However, compared to WT controls, Alb-AC mice exhibited significantly reduced blood glucose levels during the oral glucose tolerance test (OGTT) (Figure 1C), indicating an improvement in systemic glucose tolerance. Additionally, the plasma insulin levels during the OGTT were also markedly lower in the Alb-AC mice (Figure 1D). Furthermore, HFD-dox-fed Alb-AC mice also had substantially decreased blood glucose levels after insulin injection during an insulin tolerance test (ITT) (Figure 1E), suggesting enhanced insulin sensitivity.

We performed hyperinsulinemic-euglycemic clamp studies. The glucose infusion rate needed to maintain euglycemic conditions (~150 mg/dl) was increased in Alb-AC mice compared to their littermate controls (Figure 1F, left panel), demonstrating that whole-body insulin sensitivity is improved. Whole body glucose disposal was not altered (Figure S1J), suggesting minimal effects on muscle insulin action. There were no significant changes in lipolysis between WT and Alb-AC mice under clamped or under insulinopenic conditions where insulin-induced suppression of lipolysis is more pronounced (Table 1; Figure S1K). Hepatic glucose production under hyperinsulinemic clamp conditions was suppressed more efficiently in Alb-AC mice (Figure 1F, right panel). Specifically, hyperinsulinemic clamp conditions suppressed hepatic glucose production of Alb-AC mice by ~47% compared to ~15% of WT mice (Figure S1L). Insulin signaling in the liver and adipose tissue was assessed by insulin-stimulated phosphorylation of Akt. p-Akt levels were greatly increased in the liver of transgenic animals. Moreover, even though the increase in ceramidase activity is restricted to hepatocytes, insulin-stimulated p-Akt in gonadal adipose tissue was also elevated (Figure 1G). Consistent with these observations, 2-deoxyglucose uptake during clamped hyperinsulinemic conditions showed that the gonadal (gWAT), subcutaneous (sWAT), and mesenteric (mWAT) fat pads of Alb-AC mice had increased 2-deoxyglucose uptake compared to WT (Figure 1H). Differences in glucose disposal and glucose production could not be explained by minimal differences in body weight or glucose or insulin levels during the clamped state (Table 1).

Overexpression of Acid Ceramidase in the Hepatocyte Prevents HFD-Mediated Hepatic Lipid Accumulation and Attenuates Adipose Tissue Inflammation and Fibrosis

While the HFD causes a marked increase in the liver weights of both Alb-AC and WT mice, the livers from Alb-AC mice are qualitatively smaller and less lipid-laden compared to WT (Figure 2A, left). The weights of the Alb-AC mouse livers were lower than that of WT mice, even after normalizing to body weight (Figure 2A, right). Histologically, the Alb-AC liver exhibited substantially less lipid accumulation compared to that of the WT control (Figure 2B, left). Mirroring the histological findings, Alb-AC mice exhibited almost a 3-fold decrease in hepatic triglyceride (TG) content compared to WT mice (Figure 2B, right).

In addition, we performed a triglyceride clearance test by gavaging a lipid emulsion (20% Intralipid) to both Alb-AC and WT mice. Surprisingly, the Alb-AC mice peaked at higher levels and had a lower rate of triglyceride clearance compared to WT (Figure 2C). To determine whether AC overexpression affected hepatic VLDL production, mice were fasted for 3 hr followed by intravenous injection of tyloxapol, a potent inhibitor of capillary lipoprotein lipase. Alb-AC mice had higher plasma TG levels compared to WT mice 2 hr post-injection, indicating that the rate of VLDL production in Alb-AC mice is markedly higher compared to WT mice (Figure 2D).
We intravenously injected a \(^3\)H-triolein tracer into Alb-AC and WT mice that allows for assessments of lipid storage and \(\beta\) oxidation rates in different tissues. Alb-AC mice have a reduced lipid uptake in the liver compared to WT mice; concurrent increases were observed in the gonadal and brown fat pads (Figure 2E). Lipid oxidation was not significantly altered (Figure S2A). In order to determine how overexpression of AC ameliorates hepatic steatosis, we performed microarray analyses on liver tissues specifically at 2 weeks post-induction and then at 2 months post-induction of the AC transgene in the liver. A reduction of genes in fatty acid (FA) synthesis and uptake pathways was evident. qPCR analyses confirmed the reduction in FA synthesis genes (ACC, FAS, Scc-1, Elovl5, and SREBP-1a) and FA uptake genes (CD36, FATP2, FATP5, L-FABP) by the end of week 8 (Figures S2B and S2C). Surprisingly, qPCR of the fatty acid synthesis genes and fatty acid uptake genes in gWAT showed a corresponding increase during this same period (Figures S2B and S2C).

A noticeable observation was the differences in fat pad distribution in Alb-AC mice compared to WTs when challenged with HFD-dox. Alb-AC gWAT was substantially larger than the corresponding pads in WT’s after HFD-dox challenge when normalized to body weight, while the mWAT of Alb-AC was relatively smaller than its counterpart in WT littermates (Figures S2D and S2E). Furthermore, whole body NMR data show that the overall composition of fat and lean mass in Alb-AC and WT mice are comparable (Figure S2F), thus suggesting that changes in fat pad weights are the result of redistribution rather than an overall increase or decrease of overall adipose tissue mass.
We evaluated gonadal adipose tissue for histological differences (Figure 2F). While WT mice on HFD-dox displayed crown-like structures characteristic of immune infiltration, Alb-AC fat pads showed far fewer signs of inflammation. Mac-2 immunohistochemistry staining confirmed that Alb-AC fat pads had greatly reduced levels of infiltrated macrophages compared to those of WT. In addition, we found markedly less fibrosis in the fat pads of Alb-AC compared to their WT counterparts, as determined by trichrome staining (Figure 2F). Expression of inflammatory cytokines such as TNF-α, IL-6, and IL-10 were substantially reduced in the gWAT of Alb-AC mice (Figure S2G). Furthermore, fibrosis markers such as Col1α, Col3α, and Col6α were also lower in the gWAT of Alb-AC mice compared to WT (Figure S2H). We examined the levels of ceramides in the gWAT of HFD-dox-fed Alb-AC and WT mice and found that dihydro-ceramides and hexosyl-ceramide species to be significantly lowered in Alb-AC mice after HFD-dox challenge compared to WT controls, while other ceramide species remained unaltered (Figure 2G). Furthermore, there are no significant alterations in specific chain lengths of gWAT ceramides (Figure S2I). gWAT sphingoid species levels, especially sphingosine and sphinganine, were also significantly lowered in Alb-AC mice compared to WT (Figure 2H). Ceramide synthesis pathway genes (SPT2, SK1, CerS6) were found to be significantly reduced in the gWAT of Alb-AC mice compared to WT at the end of 8 weeks of HFD-dox feeding (Figure S2J). By contrast, examination by qPCR of the genes in the ceramide synthesis pathway in the Alb-AC liver showed a profound increase in several ceramide synthase (CerS) genes including CerS2, CerS5, and CerS6. In contrast, CerS1 expression was significantly reduced (Figure S2J).

### Acid Ceramidase Overexpression in White Adipose Tissue Improves Glucose Metabolism

By crossing the Tet-responsive acid ceramidase transgenic line (TRE-AC) with an adiponectin-rTA transgenic allele (Art) we were able to achieve inducible overexpression of acid ceramidase (Art-AC) within multiple WAT depots (Figure S3A). Upon doxycycline induction, AC overexpression is achieved exclusively in the fat pads and remains unchanged in other tissues, such as the liver (Figure S3B). In the absence of doxycycline, overexpression was not detected, but acid ceramidase expression rose 3.5-fold following exposure to doxycycline in the diet (200 mg/kg, 10 days, Figure S3C). This corresponded to a 1.8-, 1.3-, and 3.7-fold increase in acid ceramidase activity in sWAT, gWAT, and mWAT, respectively, without alterations in hepatic ceramidase activity (Figure S3D). Furthermore, serum ceramidase activity in WT and Art-AC were not significantly altered (Figure S3E). The lack of ceramide hydrolysis was further confirmed with C17 ceramide, showing no alterations in hepatic ceramidase activity (Figure S3F). On a doxycycline-enriched chow diet, several ceramide subspecies were significantly reduced in mWAT, while total ceramides were significantly decreased in the liver (Figures S3G and S3H). Concomitant decreases in fasting glucose and glucose excursion during oral glucose tolerance tests were evident (Figure S3I) without any changes in body weight (34.4 ± 3.1 g versus 32.7 ± 3.3 g for wild-type and Art-AC, respectively).

Art-AC transgenic mice have a marked ability to maintain normal glucose homeostasis on HFD. Total ceramides were reduced in mesenteric (67%), gonadal (40%), and subcutaneous (30%) fat pads, with C16 and C18 ceramides showing the most consistent and robust changes (Figure 3A). Serum sphingolipid species were also altered (Figure 3B). Specifically, serum levels of C20:0 ceramides were reduced to 42% of WT levels (Figure 3C). Glucose levels were lower during the fasting and 15 min following glucose administration (Figure 3D). Improvements in insulin sensitivity were confirmed by the enhanced lowering of glucose upon insulin administration (Figure 3E) and occurred without changes in body weight (40.0 ± 5.0 versus 38.4 ± 6.1 for wild-type and Art-AC, respectively). The improvements in whole-body glucose homeostasis and insulin sensitivity could hardly be explained by the relatively minor contribution of adipose to glucose disposal. To address this more specifically, we performed hyperinsulinemic-euglycemic clamps. The glucose infusion rate required to maintain euglycemia was markedly higher in Art-AC transgenic mice, confirming improvements in whole-body insulin sensitivity (Figure 3F). The kinetics of 3H-glucose disposal were not altered, suggesting negligible changes in insulin-stimulated glucose disposal by muscle (Figure S3K). Rather, Art-AC transgenic mice showed improvements in the ability of insulin to suppress endogenous glucose production (Figure 3G). Specifically, endogenous glucose production was suppressed by ~60% in Art-AC compared to ~15% in WT (Figure S3L). These improvements in insulin sensitivity could not be explained by changes in body weight, circulating insulin during the clamped state, or variations in the achieved glucose concentrations during the clamped state (Table 1). Furthermore, there were no significant changes in lipolysis between WT and Art-AC mice under clamped or insulinopenic conditions (Table 1; Figure S3M). Enhanced insulin-stimulated phosphorylation of Akt was present in livers of these mice 30 min after insulin

### Table 1. Metabolic Parameters before and during Hyperinsulinemic-Euglycemic Clamp Study in WT and AC Transgenics

<table>
<thead>
<tr>
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<th>Basal</th>
<th>Clamped</th>
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<tr>
<td><strong>Body Weight (g)</strong></td>
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<tr>
<td>WT</td>
<td>49.4 ± 1.8</td>
<td>174.2 ± 23.3</td>
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<tr>
<td>Alb-AC</td>
<td>48.8 ± 2.0</td>
<td>207 ± 2.4</td>
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<tr>
<td>WT</td>
<td>30.8 ± 1.6</td>
<td>191 ± 3.9</td>
</tr>
<tr>
<td>Art-AC</td>
<td>30.9 ± 1.06</td>
<td>174.2 ± 23.3</td>
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<tr>
<td><strong>Plasma Glucose (mg/dl)</strong></td>
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<tr>
<td>WT</td>
<td>208.2 ± 7.8</td>
<td>358.4 ± 0.4</td>
</tr>
<tr>
<td>Alb-AC</td>
<td>191 ± 3.9</td>
<td>42 ± 0.03</td>
</tr>
<tr>
<td>WT</td>
<td>207 ± 2.4</td>
<td>51 ± 0.08</td>
</tr>
<tr>
<td>Art-AC</td>
<td>174.2 ± 23.3</td>
<td>42 ± 0.03</td>
</tr>
<tr>
<td><strong>Plasma Insulin (ng/ml)</strong></td>
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<tr>
<td>WT</td>
<td>0.87 ± 0.13</td>
<td>0.59 ± 0.15</td>
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<tr>
<td>Alb-AC</td>
<td>0.83 ± 0.13</td>
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<tr>
<td>WT</td>
<td>0.98 ± 0.1</td>
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<tr>
<td>Art-AC</td>
<td>0.68 ± 0.16</td>
<td>0.42 ± 0.04</td>
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<tr>
<td><strong>Plasma NEFA (mmol/l)</strong></td>
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<tr>
<td>WT</td>
<td>0.47 ± 0.06</td>
<td>0.32 ± 0.02</td>
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<tr>
<td>Alb-AC</td>
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<tr>
<td>WT</td>
<td>0.51 ± 0.08</td>
<td>0.51 ± 0.06</td>
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<tr>
<td>Art-AC</td>
<td>0.42 ± 0.04</td>
<td>0.38 ± 0.09</td>
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<tr>
<td><strong>Plasma Glycerol (mg/ml)</strong></td>
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<tr>
<td>WT</td>
<td>0.41 ± 0.04</td>
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<tr>
<td>Alb-AC</td>
<td>0.42 ± 0.03</td>
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</tr>
<tr>
<td>WT</td>
<td>0.59 ± 0.15</td>
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<tr>
<td>Art-AC</td>
<td>0.38 ± 0.09</td>
<td>0.36 ± 0.08</td>
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*Abbreviations: HFD, high-fat diet; WT, wild-type; Alb-AC, acid ceramidase transgenic; Art-AC, acid ceramidase transgenic.*
stimulation (Figure 3H). Lowering ceramides in adipose also enhanced insulin-stimulated phosphorylation of Akt in gonadal fat pads, while changes in total Akt expression were not altered in either tissue (Figure 3H). The rate of 2-deoxyglucose uptake doubled in gonadal, mesenteric, and subcutaneous fat of Art-AC transgenic mice (Figure 3I).

Acid Ceramidase Overexpression in White Adipose Tissue Improves Hepatic Lipid Accumulation

We evaluated gonadal adipose tissue for histological differences (Figure 4A). While WT mice on HFD-dox displayed crown-like structures characteristic of immune infiltration, Art-AC fat pads showed far fewer signs of inflammation. Mac-2 immunohistochemistry staining confirmed that Art-AC fat pads had greatly reduced levels of infiltrated macrophages compared to those of WT. In addition, we found markedly less fibrosis in the fat pads of Art-AC compared to their WT counterparts, as determined by trichrome staining (Figure 4A). Correspondingly, mRNA levels of TNF-α and pro-fibrotic genes (Col1α1, Col3α1, Col6α1) were found to be notably lowered in WAT compared to WT (Figure S4A).

Considering the fact that overexpression of AC in the liver resulted in improvements in adipose tissue metabolic health, we wanted to examine if the reverse is also true. Dissection of the livers of the Art-AC transgenic mice clearly revealed less steatotic livers, judged by both overall appearance and color (Figure 4B, left). Hepatomegaly caused by a HFD is diminished by transgene overexpression in adipose tissue (Figure 4B, right). Lowering of hepatic triglyceride content was also evident histologically (Figure 4C, left). Transgene overexpression in adipose tissue...
tissue decreased hepatic triglyceride accumulation on chow diet by 45% (Figure S4B) and caused a 57% decrease in hepatic triglyceride while on HFD (Figure 4C, right).

We subsequently evaluated lipid metabolism in more detail. Neither fatty acids (Table 1) nor serum triglycerides (Figure 4D) were different during the basal state. During hyperinsulinemic clamps, Art-AC mice showed a trend toward improved suppression of lipolysis (p = 0.06), as serum fatty acids trended lower in Art-AC transgenic mice during the clamped state (Table 1). By contrast, no changes in glycerol were apparent in the clamped state. Following lipid gavage, the triglyceride clearance was not different between WT and Art-AC transgenic mice (Figure 4D). Hepatic secretion of triglyceride was not affected by adipose-restricted expression of acid ceramidase (Figure 4E).
In a complimentary approach, uptake of tritiated oleate was not significantly altered in any of the fat pads evaluated following an intravenous bolus of $^3$H-Triolein (Figure 4F). Lipid oxidation was not altered in any of the tissues evaluated (Figure S4C). Unlike adipose tissues, livers of transgenic mice displayed a 3.8-fold decrease in lipid uptake (Figure 4F). Furthermore, levels of C$_{16:0}$ and C$_{18:0}$ ceramide species were also markedly lower in Art-AC compared to WT (Figure 4G). Hepatic diacylglycerols were not significantly altered (Figure S4D).

**Adipose-Specific Acid Ceramidase Reverses Insulin Resistance and Hepatic Steatosis More Rapidly Than Liver-Specific Ceramidase Induction**

To determine whether the induction of AC in the liver or adipose is able to reverse impairments in total body glucose metabolism and rescue hepatic steatosis, Alb-AC, Art-AC, and WT mice were first fed a doxycycline-free HFD for 2 months to facilitate the development of hepatic steatosis prior to transgene induction. At the end of the 2 months, diets were switched to HFD-dox to induce AC overexpression. Approximately 1 month after transgene induction in the liver, we found C$_{16:0}$ and C$_{18:0}$ ceramide levels to be appreciably lowered along with concurrent improvements in insulin signaling, as indicated by increased phosphorylation of Akt (Figures 5A and 5B). Furthermore, hepatic lipid accumulation was significantly lowered 4 weeks after inducing overexpression of the transgene in the liver (Figure 5C). An insulin tolerance test does not reveal any changes 3 days post-induction of AC in the liver (Figure 5D). Our data not only showed that the effects of the transgene take ~1 month to result in significant physiological effects, but also that the local overexpression of AC in the liver can rescue livers with severe steatosis after the steatosis has fully developed.
Figure 5. Induction of Acid Ceramidase Significantly Improves Insulin Signaling and Rescues Hepatic Steatosis in Mice with Diet-Induced Obesity

All mice were maintained on high-fat diets for 8 weeks prior to treatment with doxycycline. (A) Liver ceramide species levels were quantified by tandem MS/MS for mice at pre-Dox treatment, 2 weeks, 4 weeks, and 8 weeks post-Dox treatment (n = 4 at each time). (B) Representative immunoblots of phosphorylated and total Akt from liver of insulin-stimulated WT and Alb-AC mice at pre-Dox treatment, at 2 weeks, 4 weeks, and 8 weeks post-Dox treatment (n = 4 at each time). (C) Liver triglycerides were quantified at different time points before or after Dox treatment (n = 4 at each time). (D) Circulating glucose levels measured during an insulin tolerance test (ITT) (0.75 U/kg) 3 days after HFD-dox treatment (n = 4). (E) Liver ceramide species levels were quantified by tandem MS/MS for mice at pre-Dox treatment, 2 weeks, 4 weeks, and 8 weeks post-Dox treatment (n = 4 at each time). (F) Representative immunoblots of phosphorylated and total Akt from liver of insulin-stimulated WT and Art-AC mice at pre-Dox treatment, at 24 hr, and 72 hr post-Dox treatment (n = 4 at each time). (G) Liver triglycerides were biochemically measured before and at the indicated time points after doxycycline addition to the diet in WT and Art-AC mice (n = 6–10 at each time). (H) Insulin tolerance tests were performed 3 days after doxycycline treatments in WT and AC-Art littermates (n = 5). *p < 0.05 by Student’s t test.

Overexpression of AC within the adipocyte offers a much more rapid rescue of insulin resistance and hepatic steatosis. C16 and C18 hepatic ceramides are substantially decreased within 3 days of treatment (Figure 5E). Western blotting for p-Akt in the liver of Art-AC mice shows improvements in p-Akt levels upon insulin administration within as short as 3 days post-induction of AC (Figure 5F). Within 3 days of transgene activation in the adipocyte, hepatic triglyceride content is lower and continues to improve substantially throughout the 4-week doxycycline treatment (Figure 5G). Remarkably, insulin-stimulated lowering of blood glucose is improved compared to wild-type littermates during insulin tolerance tests after just 3 days (Figure 5H). Collectively, these data show the ability of ceramidase to overcome previously established metabolic perturbations promoted by diet-induced obesity, with slightly different kinetics depending on whether we express AC in hepatocytes or in adipocytes.

Ceramides Influence Hepatic Lipid Uptake via PKCζ-Mediated Influence on CD36, and AC Downregulates Lipid Uptake and Fatty Acid Synthesis Genes in the Liver

Livers from Alb-AC and Art-AC mice showed comparable decreases in expression of CD36 as compared to WT littermates (Figure 6A). The relative abundance of hepatic CD36 protein was also clearly reduced in both Art-AC and Alb-AC transgenic mice (Figure 6B).

In addition to their roles as mediators of ceramide-induced insulin resistance, atypical PKCs have been noted to relay critical signals for lipogenesis and lipid uptake (Luiken et al., 2004, 2009; Sajan et al., 2004, 2009; Taniguchi et al., 2006). Consistent with this, aPKC expression (Figure 6C) and activity (Figure 6D) were decreased in the livers of Alb-AC and Art-AC transgenic mice. Furthermore, aPKC activity was significantly lowered in Art-AC mice after only three days of doxycycline treatment (Figure S5A).

Since aPKCs can play a permissive role in CD36 translocation, we evaluated the propensity for ceramide to facilitate lipid uptake. In cultured H4IIE hepatocyte cells, a 1 hr pre-incubation with the short chain ceramide analog C2-ceramide was sufficient to stimulate a 63% increase in palmitate uptake (Figure 6E) and markedly enhanced aPKC activity (Figure 6F). Incubation of cells with C2-ceramide yielded the same lipid uptake results as when cells were pretreated with palmitate, which promotes the formation of endogenous ceramides intracellularly (Figure S5B). To evaluate the requirement for aPKC in this effect, we blocked aPKC activation by including a myristoylated PKCζ.
Figure 6. Ceramides Facilitate Lipid Uptake by Mechanisms Involving Activation of PKCζ and CD36

(A) Relative abundance of CD36 mRNA was assessed by qPCR from livers of WT, Alb-AC, and Art-AC mice following 8 weeks of HFD-dox.
(B) Hepatic expression of CD36 was assessed by western blotting, and representative results are shown in duplicate.
(C) Hepatic expression of PKCζ were assessed by western blotting, and representative results are shown in duplicate.
(D) PKCζ activity was assessed from livers of WT, Alb-AC, and Art-AC mice following 8 weeks of HFD-dox.

(legend continued on next page)
pseudosubstrate inhibitor that completely prevented ceramide-induced lipid uptake, as it prevented ceramide from activating PKCζ (Figures 6E and 6F). To confirm this genetically, we achieved overexpression of dominant-negative (dn)PKCζ or expression of constitutively active (ca)PKCζ via transient transfection. Following overexpression with dnPKCζ, C2-ceramide failed to stimulate PKCζ activity in cells expressing dnPKCζ, and it also failed to stimulate lipid uptake. By contrast, overexpression of (ca)PKCζ stimulated PKCζ activity to the same degree as C2- and was sufficient to increase palmitate uptake, but could not be further enhanced by ceramide addition (Figures 6E and 6F). Immunohistological staining of H4Ile cells grown in glass bottom culture dishes showed that treatment with C2- and resulted in an increase in CD36 translocation to the membrane as judged by an increase in plasma membrane localized intensity of CD36 signal compared to cells treated with DMSO (Figure 6G). Following overexpression with dnPKCζ, C2- ceramide failed to stimulate CD36 translocation to the membrane, as there is no change between plasma membrane localized intensity of CD36 signal between C2- and DMSO-treated cells (Figure 6G). Conversely, overexpression of (ca)PKCζ in H4Ile cells resulted in an increase in CD36 translocation to the membrane, treatment with C2- ceramide of the (ca)PKCζ transfected cells did not increase the signal intensity of CD36 in the plasma membrane (Figure 6G). As sphingosine, a byproduct of the ceramidase reaction, is a known inhibitor of multiple PKCs, we also evaluated the propensity for sphingosine to alter aPKC activation and lipid uptake. Incubation of H4Ile cells with sphingosine, rather than ceramide impaired aPKC activity (Figure S5C) and diminished palmitate uptake (Figure S5D).

DISCUSSION

Here, we report mouse models demonstrating inducible decreases of ceramide within the liver and adipose. The use of an adiponectin promoter–driven rtTA (Art) has allowed us to evaluate the adipocyte-specific contributions of sphingolipids to whole-body metabolic dysfunction. Our inducible ceramide model bypasses the compensatory mechanisms and developmental issues present in constitutive models and gives us the opportunity to study acute modifications of ceramide-induced metabolic dysfunction in adult mice. With this system, we demonstrate that the acute depletion of ceramide in hepatocytes or adipocytes of adult mice can prevent and reverse the development of hepatic steatosis while simultaneously improving systemic glucose tolerance and insulin sensitivity in adult mice with diet-induced obesity. These studies highlight the prominent cross-talk between the liver and adipose tissue that allows for equilibration of sphingolipids between the two tissues. Furthermore, these studies define a causal role for ceramide in the pathogenesis of diet-induced NAFLD.

We found that that C16:0 and C18:0 ceramide levels to be 50% of WT in Alb-AC mice livers, indicating that the hepatic accumulation of particular ceramide subspecies more prominently contributes to the development of NAFLD and systemic insulin resistance. Consistent with our findings, recent work has shown that mice with ceramide synthase 6 deficiency (cerS6−/−) exhibit reduced C16:0 ceramide levels and are protected from HFD-induced obesity and glucose intolerance (Turpin et al., 2014). By contrast, heterozygous deletion of ceramide synthase 2 promotes a paradoxical increase in C16:0 ceramides to confer greater susceptibility to diet-induced insulin resistance (Rainchur et al., 2014). Importantly, we also observed that concurrent with the lowering of hepatic ceramides, hepatic DAG levels were increased in our Alb-AC mice to almost double of those measured in WT mice. In line with several studies, these findings further question the role of liver DAGs as a causative agent of hepatic steatosis and insulin resistance (Brown et al., 2010; Farese et al., 2012; Minehira et al., 2008; Monetti et al., 2007; Voshol et al., 2003). Nonetheless, this does not rule out the possibility that a particular DAG subspecies (i.e., sn-1,2-diacylglycerols) is able to induce hepatic steatosis and insulin resistance under certain conditions (Boni and Rando, 1985). Thus, these studies directly link excess accumulation of hepatic ceramides to the development of non-alcoholic fatty liver disease and systemic insulin resistance in rodents.

Elevated circulating ceramides are observed in patients with type 2 diabetes and these levels correlate with the severity of
insulin resistance (Boon et al., 2013; Haus et al., 2009; Lopez et al., 2013). Whether plasma ceramides are a contributor or merely a marker of systemic insulin resistance has been unresolved. We show that when ceramide species are acutely broken down in liver and adipose of obese mice, there is also a corresponding significant lowering of plasma ceramides. Thus, our current findings not only suggest that the liver can be a major contributor of circulating ceramide species, but also reveal that the adipocyte may also contribute substantially to changes in serum ceramides. While it appears likely that these two tissues shuttle a common pool of sphingolipids back and forth through the circulation, it remains uncertain if the adipose supplies the liver with ceramides–where they can be repackaged into lipoprotein particles. Alternatively, each of these tissues may function as a sink whereby excess circulating ceramides may be taken up from the circulation for storage or metabolism. Furthermore, these findings point to the notion that plasma ceramide levels reflect changes in hepatic ceramide levels and are valuable markers of hepatic metabolic health.

The roles of atypical PKCs as mediators of ceramide-induced insulin resistance have been previously reported. In particular, C16 ceramides are potent activators of PKCζ in vitro (Müller et al., 1995). Specifically, in cultured muscle cell lines, PKCζ can phosphorylate the plextrin homology domain of Akt on threonine residue 34 (Powell et al., 2003). This phosphorylation site alters the affinity for Akt to 3,4,5-triphosphate, thus interfering with Akt translocation to plasma membrane microdomains where it is activated (Fox et al., 2007). A recent report by the Farese group suggests that aberrant activation of atypical PKC, is evident in livers of Lepob/ob mice and is essential for impairments in Akt-mediated signaling to FoxO1; this is rescued in vivo by pharmacological inhibition of atypical PKCs (Sajan et al., 2015). PKCζ has also attracted attention for its role in lipid homeostasis, particularly as a mediator of SREBP-1c driven lipogenesis (Sajan et al., 2004, 2009; Taniguchi et al., 2006). Moreover, PKCζ has also been previously noted to play a facilitative role in CD36 activation to promote lipid uptake into cardiomyocytes (Luiken et al., 2009). We show here that targeted manipulation of ceramide alters atypical PKC activation in the liver. As such, our data support the notion that ceramides may be a causal effector of selective insulin resistance by driving atypical PKC activation and associated lipogenic and lipid uptake processes, while simultaneously impairing Akt-mediated regulation of hepatic glucose output. The contribution of sphingoid bases to this process is intriguing, as sphingosine can stimulate or suppress PKCζ activation, depending on the dosage (Müller et al., 1995). Here, we note the potential of sphingosine to impair the activation of aPKC and prevent lipid uptake. The rapid release of sphingosine from the adipocyte into the portal circulation for delivery to the liver may explain how adipose-specific ceramidase activation exerts more rapid beneficial effects on the liver.

Collectively, our data suggest that the accumulation of ceramides is critical in the development of non-alcoholic fatty liver disease and hepatic insulin resistance. Induction of AC activity rescues existing hepatic steatosis and metabolic syndrome in mice with diet-induced obesity, suggesting that it is a potential therapeutic target. Strategies for AC enzyme replacement have been under development for the treatment of Farber disease (AC deficiency) (Schuchman and Galanin, 2014), and this may provide additional benefits in the form of systemic metabolic improvements. Past clinical data have shown that serum adiponectin levels inversely correlate with hepatic triglyceride content (Turer et al., 2012). Considering that adiponectin’s main target tissue is the liver and it is known to confer ceramidase activity through its receptors AdipoR1 and R2, we expect adiponectin to exert similar effects as seen for our acid ceramidase transgene, such as reducing hepatic triglyceride accumulation and improving hepatic insulin sensitivity (Holland et al., 2011).

**EXPERIMENTAL PROCEDURES**

**Animals**

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of University of Texas Southwestern Medical Center at Dallas. TRE-AC mice were generated by subcloning the mouse AC gene into a plasmid containing the TetO element. Following linearization, the construct was injected into C57/B16-derived blastocysts. Transgene-positive offspring were genotyped using PCR with the following primer sets: TRE-AC, 5’-GACTG TATTCACACCTTGTAGT, and 5’-CATGTCATCCATTCTAAACAA. The Rosa26-loxp-stop-loxp-rTA and Albumin-Cre mice were lines were obtained from Jackson Laboratories. Albumin-Cre mice were bred with the Rosa26-loxp-stop-loxp-rTA mice to achieve liver-specific expression of rTA. This mouse was subsequently crossed to the TRE-AC transgenic mice. The resulting triple transgenic mice expressed AC in liver only after exposure to doxycycline (Dox). A transgenic mouse was generated using a 5.4 kb adiponectin promoter fragment driving the expression of rTA exclusively in adipocytes. All overexpression experiments were performed in a pure C57/B16 background. All experiments were conducted using littermate-controlled male mice. All Dox-chow diet (200 mg/kg Dox) or HFD-Dox (200 mg/kg Dox) experiments were initiated at ~6–16 weeks of age.

**Acid Ceramidase Activity Assay**

Acid ceramidase activity was determined by a fluorimetric assay using the substrate Rbm 14-12, a synthetic ceramide analog that possess a 12-carbon fatty acid chain length, at 20 μM after incubation for 3 hr with tissue lysates (20 μg protein) of both wild-type mice (WT) and Alb-AC mice after 8 weeks of HFD-dox (Bedia et al., 2010).

**Lipid Quantifications**

Sphingolipids were quantified as described previously by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC/ESI/MS/MS) using a Shimadzu Nexera X2 UHPLC system coupled to a Shimadzu LCMS-8050 triple quadrupole mass spectrometer (Holland et al., 2011). Diacylglycerol and C17 FFA were quantified using an ABI 5600+ (AB Sciex) following direct infusion of extracted lipids containing 18 mM ammonium fluoride to aid in ionization of neutrals and to reduce salt adducts. Data from the AB Sciex 5600+ was collected and calibrated with Analyst and PeakView Software (AB Sciex). Lipid species were identified based on exact mass and fragmentation patterns and verified by lipid standards.

**Streptozotocin Administration**

Mice were fasted for 6 hr and subjected to a single intraperitoneal (i.p.) injection of streptozotocin (STZ) (Sigma #S1030) at the dose of 135 μg/kg body weight. STZ was stored at -20 C as powder and freshly diluted in ice-cold sodium citrate buffer (0.1 M, pH 4.5) before injection as previously described (Ye et al., 2014).

**aPKC Inhibitor**

Inhibitor of aPKCs, PKGζ and PKCζ, 2-acetyl-1,3-cyclopentandione (ACPD), was purchased from Sigma. Its specificity was reported previously (Sajan et al., 2014). In addition, we presently found that ACPD did not inhibit kinases, Akt2, FGFR1/2/3/4, mTOR, GSK3β, IAK1/4, JAK1/2, MEK1, ERK1/2, JNK1/2, PKA, Src, ROCK2, ROS1, or PI3Kζ’s, as tested by Life
Technologies. Alb-AC and WT controls were injected subcutaneously daily with ACPD (10 mg/kg) in PBS or PBS only for 1 month. Art-AC and WT controls were injected subcutaneously daily with ACPD (10 mg/kg) in PBS or PBS only for 3 days.

**Cell Culture**

Halle rat hepatoma cells were cultured in low glucose DMEM supplemented with 10% fetal bovine serum. Dominant-negative PKC δ (Soh and Weinstein, 2003) and caPKC (Chou et al., 1998) were purchased from Addgene and transfections were performed with Lipofectamine 3000 (Invitrogen) according to the manufacturer’s instructions.

**Statistics**

All results are provided as means ± SEM. All statistical analyses were performed using GraphPad Prism. Differences between the two groups over time (indicated in the relevant figure legends) were determined by a two-way ANOVA for repeated-measures. For comparisons between two independent groups, a Student’s t test was used. Significance was accepted at p < 0.05.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2015.06.007.

**AUTHOR CONTRIBUTIONS**

J.Y.X. (liver results) and W.L.H. (adipocyte results) designed the studies, carried out the research, interpreted the results, and wrote the manuscript. C.M.K., K.S., A.X.S., M.J.P., A.J.S., J.G.M., and R.G. assisted in study design, performed research, and reviewed the manuscript. P.E.S. designed the study, analyzed the data, reviewed and revised the manuscript, and is responsible for the integrity of this work. All authors approved the final version of the manuscript.

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