The glucocorticoid-Angptl4-ceramide axis induces insulin resistance through PP2A and PKCζ

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Chronic glucocorticoid exposure is associated with the development of insulin resistance. We showed that glucocorticoid-induced insulin resistance was attenuated upon ablation of Angptl4, a glucocorticoid target gene encoding the secreted protein angiopoietin-like 4, which mediates glucocorticoid-induced lipolysis in white adipose tissue. Through metabolomic profiling, we revealed that glucocorticoid treatment increased hepatic ceramide concentrations by inducing enzymes in the ceramide synthetic pathway in an Angptl4-dependent manner. Angptl4 was also required for glucocorticoid to stimulate the activities of the downstream effectors of ceramide, protein phosphatase 2A (PP2A) and protein kinase Cζ (PKCζ). We further showed that knockdown of PP2A or inhibition of PKCζ or ceramide synthesis prevented glucocorticoid-induced glucose intolerance in wild-type mice. Moreover, the inhibition of PKCζ or ceramide synthesis did not further improve glucose tolerance in Angptl4−/− mice, suggesting that these molecules were major downstream effectors of Angptl4. Overall, our study demonstrates the key role of Angptl4 in glucocorticoid-augmented hepatic ceramide production that induces whole-body insulin resistance.

INTRODUCTION
Insulin resistance is a major risk factor for type 2 diabetes and cardiovascular diseases. Chronic and/or excess glucocorticoid exposure has been associated with the development of insulin resistance (1–3). Glucocorticoids reduce insulin-stimulated glucose utilization in skeletal muscle and white adipose tissue (WAT). Glucocorticoid exposure also suppresses insulin responsiveness in the liver and potentiates glucose homeostasis and insulin actions (6, 7, 10). A final mechanism is that glucocorticoid treatment increases hepatic ceramide concentrations. One line of evidence supporting this mechanism is that inhibition of ceramide synthesis by the small-molecule inhibitor myriocin compromises glucocorticoid-induced insulin resistance (11). Also, the ability of glucocorticoids to cause insulin resistance is reduced in mice lacking Des2, an enzyme in the ceramide biosynthesis pathway (11).

The biological responses of glucocorticoids are mainly mediated by the glucocorticoid receptor, which directly regulates its primary target genes. Therefore, to understand the molecular mechanism of glucocorticoid-induced insulin resistance, the first step is to identify glucocorticoid receptor primary target genes that participate in this glucocorticoid-regulated process. We have previously identified Angptl4 (which encodes angiopoietin-like 4) as a glucocorticoid receptor primary target gene in hepatocytes and adipocytes (12, 13). The Angptl4 gene encodes a secreted protein that promotes lipolysis in adipocytes (14) and inhibits extracellular lipoprotein lipase (LPL) (15, 16). Angptl4−/− mice display impaired glucocorticoid-induced WAT lipolysis (14) and are therefore excellent models for assessing the role of WAT lipolysis in glucocorticoid-modulated metabolite changes in peripheral tissues that cause insulin resistance.

Here, we analyzed the effects of glucocorticoids on glucose homeostasis and insulin actions in Angptl4−/− mice. We also performed metabolomics in the liver and skeletal muscle of Angptl4+/+ mice (which will be called wild-type mice) and Angptl4−/− mice treated with or without glucocorticoids. Our goal was to establish Angptl4 as a glucocorticoid receptor primary target gene that potentially links glucocorticoid-promoted WAT lipolysis to the changes in insulin resistance–inducing metabolites in the liver and/or skeletal muscle and to elucidate the mechanism governing this process.

RESULTS
Glucocorticoid-induced insulin resistance was improved in Angptl4−/− mice
Wild-type and Angptl4−/− mice were treated with or without dexamethasone (a synthetic glucocorticoid) for 7 days. Intraportal glucose tolerance tests (IPGTTs) showed that in the absence of dexamethasone treatment, there was no significant difference in basal glucose tolerance between wild-type and Angptl4−/− mice (Fig. 1, A and B). Dexamethasone treatment induced glucose intolerance in wild-type mice, as expected, but not in Angptl4−/− mice (Fig. 1, A and B). Furthermore, fasting plasma insulin concentrations were markedly higher in dexamethasone-treated wild-type mice than those in untreated wild-type and Angptl4−/− mice or in dexamethasone-treated Angptl4−/− mice (Fig. 1C, 0 min). These results confirmed that dexamethasone treatment in mice caused insulin resistance resulting in hyperinsulinemia. Moreover, the decreased glucose tolerance in dexamethasone-treated wild-type mice despite the presence of hyperinsulinemia suggested that pancreatic islet β cells could not compensate for insulin resistance. In untreated wild-type mice.
and Angptl4−/− mice, plasma insulin concentrations were increased 15 min after glucose administration but returned to basal values within 30 min (Fig. 1C). In dexamethasone-treated wild-type mice, plasma insulin concentrations were similar at all three time points measured (Fig. 1C). In dexamethasone-treated Angptl4−/− mice, plasma insulin concentrations remained high 30 min after glucose administration (Fig. 1C). In dexamethasone-treated wild-type mice, plasma insulin concentrations were increased over the course of the experiment (Fig. 1, F and G). In contrast, plasma glucose concentrations in PBS- and dexamethasone-treated control mice over the course of the experiment (Fig. 1, F and G). In contrast, plasma glucose concentrations in PBS- and dexamethasone-treated control mice over the course of the experiment (Fig. 1, F and G).

We next performed PTTs to assess the effect of glucocorticoids on hepatic gluconeogenesis. After pyruvate injection, dexamethasone-treated wild-type mice had higher plasma glucose concentrations than control mice over the course of the experiment (Fig. 1, F and G). In contrast, plasma glucose concentrations in PBS- and dexamethasone-treated wild-type mice were similar after pyruvate injection (Fig. 1, F and G). These results demonstrated that the ability of glucocorticoids to stimulate hepatic gluconeogenesis was attenuated in mice lacking Angptl4.

To identify the organ that contributes to the insulin sensitivity observed upon Angptl4 depletion, we monitored the activity of Akt in epididymal WAT (eWAT), liver, and gastrocnemius muscle after 10 min of insulin treatment in control and dexamethasone-treated wild-type and Angptl4−/− mice. Akt is phosphorylated at Ser473 and Thr308 upon insulin treatment, and we monitored phosphorylation of Thr308 and Ser473. These results demonstrated that the ability of glucocorticoids to stimulate Akt activation in eWAT and liver muscle and to attenuate Akt activation in gastrocnemius muscle. In contrast, insulin treatment increased the phosphorylation of Akt in these tissues.

**Fig. 1.** Dexamethasone-induced glucose, insulin, and pyruvate intolerance were improved in Angptl4−/− mice. Wild-type (WT) and Angptl4−/− mice were treated with phosphate-buffered saline (PBS) or dexamethasone (Dex). (A) GTT was performed after 16 hours of fasting. (B) Relative area under curve (AUC) for GTT results (relative to PBS-treated WT mice) is shown. Error bars represent SEM. n = 5 to 7 mice per group. *P < 0.05. (C) Plasma insulin concentrations were measured before glucose injection (0 time point), and 15 and 30 min after glucose injection. Error bars represent SEM. n = 5 to 7 mice per group. * indicates significant (P < 0.05) effect of dexamethasone (compared to PBS) at the given time point, ** indicates significant difference (P < 0.05) between WT dexamethasone and Angptl4−/− dexamethasone at the given time point, and *** indicates significant (P < 0.05) difference between time points. (D) ITT was performed, and results are displayed as percentage of initial plasma glucose concentrations at different time points. (E) Relative AUC for ITT results (relative to PBS-treated WT mice) is shown. Error bars represent SEM. n = 3 to 5 mice per group. *P < 0.05.
of Akt in the liver and gastrocnemius muscle of *Angptl4*−/− mice, whether they were untreated or given dexamethasone (Fig. 2, A and B). Thus, in the absence of *Angptl4*, insulin still activated Akt in the liver and gastrocnemius muscle even in the presence of dexamethasone. For eWAT, insulin treatment increased the phosphorylation of Akt in both control and dexamethasone-treated wild-type or *Angptl4*−/− mice (Fig. 2C). These results demonstrated that dexamethasone treatment induced insulin resistance in the liver and skeletal muscle of wild-type mice, an effect that was substantially reversed in *Angptl4*−/− mice. In contrast, dexamethasone treatment in eWAT had more complex effects on insulin signaling because insulin-stimulated Akt activation was present both under basal conditions and upon dexamethasone treatment. However, maximal Akt activation was somewhat reduced.

**Metabolomic profiling showed alterations of lipid metabolites in the gastrocnemius muscle and liver in control and dexamethasone-treated wild-type and *Angptl4*−/− mice**

We hypothesized that *Angptl4* was involved in glucocorticoid-induced insulin resistance by mobilizing fatty acids from WAT which were then converted in the liver to metabolites that modulated insulin action. To test this model, we performed single reaction monitoring (SRM)-based targeted metabolomic analysis to quantify the concentrations of about 150 common lipid metabolites (data file S1). We focused on the gastrocnemius muscle and liver because they became insulin-resistant upon dexamethasone treatment in our experimental system (Fig. 2, A and B). We found that dexamethasone treatment significantly increased the amounts of 11 metabolites and decreased the amounts of 48 metabolites in the liver of wild-type mice (Fig. 3A and data file S1). Surprisingly, none of the lipid species identified in muscle tissues were significantly increased after dexamethasone treatment in wild-type mice, although nine metabolite species were reduced (data file S1); none of these nine metabolites had previously been associated with the development of insulin resistance.

Six of the 11 metabolites that were increased in wild-type mouse liver by dexamethasone treatment were significantly lower in dexamethasone-treated *Angptl4*−/− mice. These were C18:0 ceramide, C16:0 sphingosine phosphate (S1P), C16:0/C18:1/C16:0 triacylglycerol (TAG), C18:0/C18:1/C18:0 TAG, C18:0/C18:1 diacylglycerol (DAG), and C16:0/C18:1 phosphatidylethanolamine (PE) (Fig. 3B). Although the amounts of ceramides, DAG (18, 19), and S1P (20, 21) have been correlated with the development of insulin resistance, only ceramides have been linked to the glucocorticoid-induced modulation of insulin sensitivity (11).

We performed liver histology to visualize the morphological changes of hepatocytes after dexamethasone treatment. For wild-type mice, dexamethasone treatment increased the size of hepatocytes, in which the unstained areas were likely due to the accumulation of triglycerides (fig. S2A). Dexamethasone treatment also increased the size of hepatocytes in *Angptl4*−/− mice (fig. S2A). However, hepatocytes in dexamethasone-treated *Angptl4*−/− mice were smaller than those in dexamethasone-treated wild-type mice (fig. S2A). This finding agrees with reduced induction of triglyceride concentrations in *Angptl4*−/− mouse liver (Fig. 3, A and B). We did not observe statistically significant differences in eWAT weight between control and dexamethasone-treated wild-type and *Angptl4*−/− mice (fig. S2B). This result is likely due to the concomitant stimulation of triglyceride synthesis and lipolysis by dexamethasone in WATs (13, 22).

**Activation of the hepatic ceramide synthetic program was attenuated in *Angptl4*−/− mice**

Distinct ceramide species, which are defined by their fatty acyl chain length, can exert specific biological functions (23, 24). Therefore, we expanded our initial SRM-based targeted metabolomic analysis by analyzing multiple ceramide species to determine whether their abundance in the liver was modulated...
by dexamethasone treatment. Sixteen of the ceramide species were similar in abundance between control wild-type and Angptl4−/− mice (Fig. 4A). However, dexamethasone treatment markedly increased the amounts of a subset of ceramide species, including C16:0, C18:0, C20:0, C20:1, C20:2, C22:1, C22:2, C24:2, and C26:0 ceramides in wild-type mouse liver (Fig. 4A). These ceramide species were lower in abundance in the livers of dexamethasone-treated Angptl4−/− mice (Fig. 4A). C24:1 ceramides were the only species that were reduced by dexamethasone treatment in the livers of wild-type mice (Fig. 4A), a decrease that was more pronounced in dexamethasone-treated wild-type mice (Fig. 4C). In dexamethasone-treated WT mice, plasma palmitate concentrations were 27% lower in abundance in dexamethasone-treated WT mice liver but not in dexamethasone-treated Angptl4−/− mice are shown. Error bars represent SEM. n = 4 mice per group. *P < 0.05.

Although dexamethasone treatment increased fatty acid flux from WAT to the liver, only 11 lipid species were increased by glucocorticoids in the livers of wild-type mice. This observation suggested that, in addition to increasing the availability of hepatic fatty acids, dexamethasone may stimulate specific metabolic pathways that regulate ceramide synthesis. Therefore, we tested this idea by analyzing the expression of genes encoding enzymes involved in ceramide synthesis. In agreement with previous observations, we found that Spt2, Cers3, Cers4, Cers5, and Cers6, which are genes encoding enzymes in the de novo ceramide synthetic pathway, were all induced by dexamethasone treatment (Fig. 4D and fig. S1) (11). The expression of Smpd1, which encodes an enzyme that converts sphingomyelins to ceramides (Fig. 4E), was also increased by dexamethasone (Fig. 4D and fig. S1). Dexamethasone also increased the expression of Sgns1, which encodes an enzyme that converts ceramides to sphingomyelins (Fig. 4E). Counterintuitively, the induction of Sgns1 and Smpd1 by dexamethasone promotes the bidirectional interconversion of ceramides and sphingomyelins, reminiscent of the effect of glucocorticoid in both promoting hepatic glycogen synthesis and gluconeogenesis (2, 26). Because we observed decreased levels of sphingomyelins (C16:0 and C18:0) upon dexamethasone treatment (Fig. 3A), we postulated that induction of Smpd1 likely dominates over the Sgns1 induction by dexamethasone. Finally, the stimulation of Spkh1 expression, a gene that encodes an enzyme that converts sphingosine to S1P (Fig. 4E), likely explains the decreased sphingosine and increased S1P amounts in the livers of dexamethasone-treated wild-type mice (Fig. 3A).

In Angptl4−/− mice, the ability of dexamethasone to augment the expression of Cers3, Cers4, Cers5, Cers6, and Spkh1 was impaired, whereas the induction of Spt2 and Smpd1 by dexamethasone was not affected.
Fig. 4. Dexamethasone-activated hepatic ceramide synthetic program was attenuated in Angptl4−/− mice. WT and Angptl4−/− mice were treated with PBS or dexamethasone for 7 days. (A to C) The concentrations of 16 different ceramide species in the liver (A), 5 different free fatty acids (FFAs) in plasma (B), and 4 ceramide species in plasma (C) of these mice were measured. n = 4 mice per group. (D) The expression of genes encoding enzymes involved in ceramide production was monitored using quantitative polymerase chain reaction (qPCR). The heat map shows the relative expression compared to that in the WT PBS group. Red shading on the heat map indicates higher expression, and green shading represents lower expression. The changes in fold induction are shown in fig. S1. n = 16 mice per group. (E) Schematic representation of ceramide synthesis pathways. The genes and metabolites that were induced by dexamethasone in WT mice liver are shown in red. The metabolites that were reduced by dexamethasone are shown in green. (F) The abundance of Cers5 and Cers6 proteins was monitored using Western blot. The bar graph represents the average intensity of bands normalized to those for Gapdh. Error bars represent SEM. n = 3 to 4 mice per group. *P < 0.05.
Angptl4

metason in the liver sofwild-type mice but not in Angptl4−/− mice (Fig. 4E). Overall, these results suggested that the reduction in ceramide production in the livers of Angptl4−/− mice was due to both diminished substrate availability and impairment in the induction of ceramide synthetic enzymes by dexamethasone (Fig. 4E). To confirm that the gene expression changes were reflected at the protein level, we performed immunoblotting for Cers5 and Cers6 as representative enzymes in ceramide changes were reflected at the protein level, we performed immunoblotting for Cers5 and Cers6 as representative enzymes in ceramide metabolism (Fig. 4E).

The activation of downstream signaling effectors by ceramides was impaired in dexamethasone-treated Angptl4−/− mice

Protein phosphatase 2A (PP2A) and protein kinase Cζ (PKCζ) act downstream of ceramide-initiated signaling (27, 28). To estimate the PP2A activity, we measured dephosphorylation of threonine-phosphopeptides using immunoprecipitates of PP2A from liver lysates. We found that dexamethasone treatment increased PP2A activity in the livers of wild-type mice (≈1.8-fold), an effect that was reduced in those of Angptl4−/− mice (Fig. 5A). We found that autophosphorylation of Thr560 of PKCζ, which is required for PKCζ activation (29), was increased by dexamethasone treatment in the livers of wild-type mice, an effect that was reduced in those of Angptl4−/− mice (Fig. 5B). These results validated the concept that dexamethasone treatment stimulates ceramide-initiated signaling in the liver, which is impaired in the absence of Angptl4.

To test whether PP2A is involved in glucose intolerance induced by glucocorticoids, wild-type and Angptl4−/− mice were infected with adenovirus expressing scrambled (Scr) shRNA or shRNA targeting Ppp2ca and treated with dexamethasone for 7 days. Relative AUC for GTT results (relative to PBS-treated WT mice) is shown. Error bars represent SEM. n = 3 to 4 mice per group. *P < 0.05. (C) GTT was performed after a 6-hour fast on WT and Angptl4−/− mice infected with adenovirus expressing scrambled (Scr) shRNA or shRNA targeting Ppp2ca and treated with dexamethasone for 7 days. Relative AUC for GTT results (relative to PBS-treated WT mice) is shown. Error bars represent SEM. n = 3 to 4 mice per group. *P < 0.05. (D) WT and Angptl4−/− mice were treated with dexamethasone. ACPD was injected into mice starting on day 4. GTT was performed at day 7 after a 6-hour fast. Relative AUC for GTT results (relative to PBS-treated WT mice) is shown. Error bars represent SEM. n = 3 to 4 mice per group. *P < 0.05.
To evaluate the role of PKCζ in glucocorticoid-induced glucose intolerance, wild-type and Angptl4−/− mice were injected with or without 2-acetyl-1,3-cyclopentanedione (ACPD), an inhibitor of atypical PKCζ, PKCζ, and PKCα (30, 31) for the final 4 days of the 7-day dexamethasone course, which reduced PKCζ activity (fig. S3C). IPGTTs showed that ACPD treatment significantly improved glucose tolerance in dexamethasone-treated wild-type but not in Angptl4−/− mice (Fig. 5D). Overall, these results suggested that both PP2A and PKCζ are involved in dexamethasone-induced glucose intolerance.

The inhibition of ceramide synthesis by myriocin reduces dexamethasone-induced insulin resistance in wild-type but not in Angptl4−/− mice

Inhibiting ceramide synthesis by the Spt1 and Spt2 inhibitor myriocin (32) reduces dexamethasone-induced insulin resistance (11). If the major role for Angptl4 in dexamethasone-induced insulin resistance is to increase hepatic ceramide production, we hypothesized that blocking ceramide synthesis would improve insulin sensitivity in dexamethasone-treated wild-type but not in dexamethasone-treated Angptl4−/− mice. Consistent with our model, we found that treatment with the ceramide synthase inhibitor myriocin attenuated dexamethasone-induced glucose intolerance in wild-type mice but not in Angptl4−/− mice (Fig. 6A).

We also monitored the activity of PKCζ to validate our hypothesis that the effect of myriocin was mediated through ceramide generation. We focused on PKCζ because reducing PKCζ activity, similar to myriocin treatment, did not further improve glucose tolerance in dexamethasone-treated Angptl4−/− mice (Fig. 5D). Myriocin treatment markedly decreased the phosphorylation of PKCζ in dexamethasone-treated wild-type mice (Fig. 6B). These results suggested that myriocin treatment reduced the ceramide-initiated signaling that was induced by dexamethasone. In contrast, in dexamethasone-treated Angptl4−/− mice, myriocin treatment did not affect PKCζ phosphorylation (Fig. 6B). These results agreed with the lack of effect of myriocin on glucose tolerance in dexamethasone-treated Angptl4−/− mice (Fig. 6B).

DISCUSSION

The molecular mechanisms underlying the antagonistic effect of glucocorticoids on whole-body insulin sensitivity are not clear. Our present studies demonstrated that Angptl4, a primary target gene of glucocorticoid receptor in hepatocytes and adipocytes (12), plays a key role in glucocorticoid-induced insulin resistance. We have previously shown that Angptl4 is required for glucocorticoid-induced WAT lipolysis and that purified Angptl4 proteins can directly enhance lipolysis in primary adipocytes (14). Here, we showed that Angptl4 was required for glucocorticoid-induced ceramide production and ceramide-initiated signaling in the liver. On the basis of these results, we propose that Angptl4 participates in glucocorticoid-induced insulin resistance by promoting lipolysis in WAT, which mobilizes fatty acids that are taken up by the liver for ceramide production (Fig. 7). In addition to promoting adipocyte lipolysis, Angptl4 also inhibits extracellular LPL (15, 16). Our present studies mainly focused on the lipolytic effect of Angptl4 in glucocorticoid-induced insulin resistance. However, we cannot exclude a role for the inhibitory effect of Angptl4 on LPL in regulating insulin sensitivity. Reducing LPL activity may lead to hypertriglyceridemia, which could also contribute to insulin resistance.

The simplest model for Angptl4 action in glucocorticoid-induced insulin resistance is that enhancing WAT lipolysis provides the precursors, such as palmitate, for hepatic ceramide production. On the basis of our results, Angptl4 action also provided signals needed for glucocorticoids to activate ceramide synthetic pathways in the liver, because without Angptl4, the ability of dexamethasone to stimulate specific ceramide synthetic genes was attenuated (Fig. 7). Glucocorticoid treatment increases the expression of several genes involved in ceramide synthesis in the liver (11) through unknown mechanisms. Chromatin immunoprecipitation sequencing analysis in mouse liver has identified glucocorticoid receptor binding sites in or nearby genomic regions of several ceramide synthetic genes, such as Cers6, Cers3, and Spt2 (33). However, whether these genes are indeed glucocorticoid receptor primary target genes would require further study. In addition to the direct activation of ceramide synthetic genes by glucocorticoid receptor, another potential mechanism is that the fatty acids generated by Angptl4-induced lipolysis in WAT provide the signals to act with glucocorticoids to regulate ceramide synthetic genes. Saturated fatty acids activate nuclear factor κB (NFκB) to stimulate ceramide synthetic pathways.
C16:0 ceramides, are increased. Furthermore, increasing acid ceramidase heterozygous mice, the amounts of C16:0 ceramides and the abundance of concentrations, protects mice from high-fat diet deletion of species of ceramides, and various studies indicate that different ceramide in the development of insulin resistance (18, 19), and the amounts of C18:0/18:1 DAG are positively correlated with homeostatic model assessment–insulin resistance (45). In contrast, SIP inhibits insulin action through SIP receptor 2 (SIP2) in the liver (20, 21). Note that our results have not identified the specific ceramide species and/or metabolites involved in glucocorticoid-induced insulin resistance. The potential role of Cers3–6 and Sphk1 on glucocorticoid-induced insulin resistance remains to be established.

Although PKCζ and PP2A are downstream of ceramides, their roles in glucocorticoid-induced insulin resistance have not been examined. Here, we showed that both were involved in glucose intolerance induced by dexamethasone treatment. Reducing PP2A abundance but not PKCζ activity further improved glucose tolerance in dexamethasone-treated Angptl4−/− mice, suggesting that the stimulation of PKCζ in the liver by glucocorticoids mainly required Angptl4, whereas there are other glucocorticoid-regulated mechanisms that can activate PP2A. It is unclear whether PKA and PKCζ act in the same or the parallel pathway to modulate insulin actions.

The increase in ceramide concentrations in the liver explains the role of Angptl4 in glucocorticoid-induced hepatic insulin resistance. However, insulin response in the gastrocnemius muscle was also improved in Angptl4−/− mice. It is surprising that in the gastrocnemius muscle, only nine metabolites were affected by dexamethasone treatment, and none of them have been previously linked to insulin sensitivity. It is possible that the metabolites modulated by dexamethasone treatment to induce insulin resistance in the gastrocnemius muscle were not in the list of target metabolomics experiments we conducted. Notably, ceramides are mainly associated with very-low-density lipoprotein (VLDL) in plasma (46), and plasma ceramide concentrations have been negatively associated with insulin sensitivity (47, 48). Therefore, it is also possible that ceramides produced in the liver are mobilized to the gastrocnemius muscle to inhibit insulin action (49). This model is somewhat supported by our observation that plasma ceramide concentrations were augmented by dexamethasone in wildtype but not in Angptl4−/− mice (Fig. 4C).

High-fat diet feeding causes inflammation in WAT. Secretion of interleukin-6 from macrophages in WAT increases, which promotes WAT lipolysis and in turn induces hepatic insulin resistance (50). Although the induction of WAT lipolysis is also involved in the glucocorticoid-induced development of insulin resistance, glucocorticoid exposure actually suppresses, rather than promotes, inflammation in WAT (51). Thus, the glucocorticoid-induced increase in Angptl4 expression, rather than cytokine production, may be the key step in glucocorticoid-induced insulin resistance. However, Angptl4 is unlikely to be the only mediator of the suppressive effect of glucocorticoids on insulin action, and a network of glucocorticoid primary target genes are likely needed to exert the effect of glucocorticoids on whole-body insulin sensitivity. Regardless, our studies suggest that Angptl4 plays a critical role in triggering interorgan communication between the WAT and the liver, leading to the suppression of insulin action. Overall, these results fill an important gap in our understanding of the metabolic functions of glucocorticoid. Furthermore, targeting Angptl4 may be a promising strategy to dissociate the beneficial anti-inflammatory effects of glucocorticoids from adverse effects such as insulin resistance.

**METHODS**

**Animals**

Angptl4−/− mice were provided by the laboratories of A. Nagy (Samuel Lunenfeld Research Institute, Mount Sinai Hospital)
J. Gordon (Washington University) (52). *Angptl4*−/− mice were generated on a mixed B6:129 Sv background. *Angptl4*+/− mice (wild-type mice) were the littermates of *Angptl4*−/− mice. Male (7 to 12 weeks old) mice were used in this study. The genotyping method was performed as previously described (52). To test insulin signaling, we injected the mice with insulin (1 U per body weight) for 10 min, and Western blot analysis was performed on various tissues. The Office of Laboratory Animal Care at the University of California, Berkeley, approved all the animal experiments (AUP-2014-08-6617).

**Drug administration**

Male (7 to 12 weeks old) *Angptl4*+/− (wild-type) and *Angptl4*−/− mice were treated with dexamethasone (−0.42 mg/kg) for 7 days through drinking water. Water-soluble dexamethasone (Sigma, D2915) was dissolved in PBS (25 mg/mL). Notably, water-soluble dexamethasone contains 65 mg of dexamethasone per gram of powder. We prepared drinking water that contains 0.0025 g of dexamethasone per liter and based on our estimate that a 30-g mouse drinks about 3.5 to 5 ml of water per day. In myriocin experiments, myriocin (0.5 mg/kg body weight) was dissolved in PBS and was injected intraperitoneally to mice on the last 4 days of dexamethasone treatment. For ACPD (Sigma, A155) treatment, mice were injected subcutaneously with ACPD (10 mg/kg) dissolved in PBS for 4 days.

**IPGTT, ITT, and PTT**

Mice were fasted for 6 or 16 hours for GTT and PTT. Mice were injected with glucose (1 g/kg body weight), insulin (1 U/kg body weight, Sigma, I0516-5ML), or sodium pyruvate (1 mg/kg body weight, Spectrum Chemical, SO193) intraperitoneally. Blood samples (one drop from tail vein) were obtained at the 0-, 30-, 60-, 90-, and 120-min time points to measure glucose levels using CONTOUR Blood Glucose Monitoring System (Contour, Bayer). Blood was also collected during different time points of GTT for measuring plasma insulin concentrations.

**Lipidomic profiling**

Liver tissues and gastrocnemius muscle tissues were used for lipidomic profiling. Lipid metabolites were extracted in 4 ml of a 2:1:1 mixture of chloroform/methanol/Tris buffer with inclusion of internal standards C12:0 dodecylglycerol (10 nmol) and pentadecanoic acid (10 nmol). Organic and aqueous layers were separated by centrifugation at 1000g for 5 min, and the organic layer was collected, dried down under N2, and dissolved in 120 µl of chloroform, of which 10 µl was analyzed by both SRM-based liquid chromatography–tandem mass spectrometry (LC-MS/MS) or untargeted LC-MS. LC separation was achieved with a Luna reverse-phase C5 column (50 mm × 4.6 mm with 5-µm-diameter particles, Phenomenex). Mobile phase A was composed of a 95:5 ratio of water/methanol, and mobile phase B consisted of a 60:35:5 ratio of 2-propanol/methanol/and water. Solvent modifiers (0.1% formic acid with 5 mM ammonium formate and 0.1% ammonium hydroxide) were used to assist ion formation and to improve the LC resolution in both positive and negative ionization modes, respectively. The flow rate for each run started at 0.1 ml/min for 5 min to alleviate backpressure associated with injecting chloroform. The gradient started at 0% B and increased linearly to 100% B over the course of 45 min with a flow rate of 0.4 ml/min, followed by an isocratic gradient of 100% B for 17 min at 0.5 ml/min before equilibrating for 8 min at 0% B with a flow rate of 0.5 ml/min.

MS analysis was performed with an electrospray ionization source on an Agilent 6430 QQQ LC-MS/MS. The capillary voltage was set to 3.0 kV, and the fragmentor voltage was set to 100 V. The drying gas temperature was 350°C, the drying gas flow rate was 10 liters/min, and the nebulizer pressure was 35 psi. Representative metabolites were quantified by SRM of the transition from precursor to product ions at associated collision energies. Data were normalized to the internal standards and also external standard curves of metabolite classes against the internal standards, and concentrations were calculated as relative metabolite concentrations compared to controls. These internal standards were added alongside dodecylglycerol and pentadecanoic acid in the 2:1:1 chloroform/methanol/Tris buffer mixture.

**Western blot**

The protein concentrations of samples were measured using bicinchoninic acid (BCA) protein assay (Thermo Scientific, 23228). Proteins (~30 µg) were mixed with 1X NuPAGE LDS Sample Buffer (ThermoFisher, NP0007) and 1X NuPAGE Sample Reducing Agent (ThermoFisher, NP0009), boiled for 5 min before applying to SDS–polyacrylamide gel electrophoresis. The following are the antibodies we used in this study: anti-Gapdh (Santa Cruz, sc-25778), anti-β-actin (Cell Signaling, 9272s), anti-phospho-Akt (Cell Signaling, 9275s), anti-Cers5 (Life Technologies, PA-520570), anti-Cers6 (Santa Cruz, sc-100554), anti-β-actin (Santa Cruz, sc-47778), anti-PKCζ (Santa Cruz, sc-216), anti-phospho-PKCζ (T410, Cell Signaling, 2060S), anti-Ppp2ca (Cell Signaling, 2041S). The intensity of the bands was quantified using ImageJ software (National Institutes of Health) and normalized to Gapdh or β-actin.

**PP2A activity assay**

The PP2A activity in the liver lysate was detected using PP2A Immunoprecipitation Phosphatase Assay kit (Millipore, 17-313FR) following the manufacturer’s protocol.

**Real-time qPCR**

Total RNA was isolated from liver tissues using TRIzol reagent (Invitrogen, 15596018). Reverse transcription was performed as follows: 0.5 µg of total RNA, 4 µl of 2.5 mM dNTP, and 2 µl of 15 µM random primers (New England Biolabs, S1254S) were mixed at a volume of 16 µl and incubated at 70°C for 5 min. A 4-µl cocktail containing 25 µl of Moloney Murine Leukemia Virus (M-MuLV) Reverse Transcriptase (New England Biolabs, M0253S), 10 U of RNasin Plus (Promega, N261B), and 2 µl of 10X M-MuLV Reverse Transcriptase Reaction Buffer (New England Biolabs, B0253S) were added, and samples were incubated at 42°C for 1 hour and then at 95°C for 5 min. The cDNA was diluted and used for real-time qPCR using the Power Eva qPCR SuperMix Kit (Biochain, K5057400) following the manufacturer’s protocol. qPCR was performed on the StepOne PCR System (Applied Biosystems) and analyzed with the DeltaCt method, as supplied by the manufacturer (Applied Biosystems). *Rpl19* gene expression was used for internal normalization. Primer sequences used in this study are listed in table S1.

**Adenovirus**

Adenovirus expressing scramble shRNA or shRNA targeting *Ppp2ca* was purchased from Vector Biolabs. The targeting sequences for *Ppp2ca* are CGACGAGGTGTTAAGGAAATA, which has been previously reported (53). Mice were injected by tail vein with 1 × 10⁸ plaque-forming units (pfu) adenovirus/mouse.
Histology
Livers from treated mice were harvested and fixed in 10% neutral buffered formalin (Sigma, HT501128). Liver was embedded, sectioned, and stained for hematoxylin and eosin by the University of California, San Francisco (UCSF) Liver Center Pathology and Imaging Core (San Francisco, CA). Images were taken at the UC Berkeley Biological Imaging Facility (Berkeley, CA) using the Zeiss AxiolImager M1 at ×20 magnification. Each image shows a portion of the hepatic lobule. One image from each treatment group was shown. At least two animals were analyzed for each treatment group. Multiple sections were performed for each liver.

Statistics
Data are expressed as SEM for each group, and comparisons were analyzed by Student’s t test.

SUPPLEMENTARY MATERIALS
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Fig. S1. Expression analysis of genes involved in ceramide metabolism.

REFERENCES AND NOTES


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The glucocorticoid-Angptl4-ceramide axis induces insulin resistance through PP2A and PKCζ

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Using glucocorticoids without insulin resistance

Inflammation can be reduced with chronic glucocorticoid treatment, which unfortunately is associated with the development of insulin resistance. Glucocorticoids induce the production of the secreted protein Angptl4 and of ceramides, a family of lipid mediators. Chen et al. showed that Angptl4 was necessary to induce the expression of genes encoding factors involved in ceramide synthesis in the livers of glucocorticoid-treated mice. Furthermore, Angptl4 was required to stimulate the activity of two downstream effectors of ceramides: PP2A and PKCζ. These results suggest that inhibiting this pathway may alleviate the insulin resistance that occurs with chronic glucocorticoid treatment.
Supplementary Materials for

The glucocorticoid-Angptl4-ceramide axis indues insulin resistance through PP2A and PKCζ

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The PDF file includes:

Fig. S1. Expression analysis of genes involved in ceramide metabolism.
Fig. S2. Liver histology and WAT weight in dexamethasone-treated wild-type and Angptl4−/− mice.
Fig. S3. PP2A abundance and PKCζ activity in mice infected with adenovirus expressing Ppp2ca shRNA or treated with a PKCζ inhibitor.
Table S1. qPCR primer list.

Other Supplementary Material for this manuscript includes the following:
(available at www.sciencesignaling.org/cgi/content/full/10/489/eaa7905/DC1)

Data file S1 (Microsoft Excel format). Lipidomic data in the liver and gastrocnemius muscle of control or dexamethasone-treated wild-type and Angptl4−/− mice.
Fig. S1. Expression analysis of genes involved in ceramide metabolism.

WT and Angptl4−/− mice were treated with PBS or 0.42 mg/kg of dexamethasone (Dex) for 7 days. Hepatic gene expression patterns were determined by RT-qPCR. Error bars represent S.E.M., n=16 mice per group, and *p < 0.05.
Fig. S2. Liver histology and WAT weight in dexamethasone-treated wild-type and Angptl4−/− mice.

WT and Angptl4−/− mice were treated with PBS or 0.42 mg/kg of dexamethasone for 7 days prior to the collection of liver and eWAT. (A) Livers were fixed in 10% neutral buffered formalin and histology was performed by the UCSF Liver Center Pathology and Imaging Core (San Francisco, CA). For each mouse liver, multiple sections were prepared. Two animals per treatment group were processed for histological analysis. Representative images for each treatment group are
shown. Scale bar (red, bottom left of top panel) represents 60 microns. (B) The graph shows the ratio of eWAT weight to total body weight. N=5-6 mice per group.

**Fig. S3.** PP2A abundance and PKCζ activity in mice infected with adenovirus expressing *Ppp2ca* shRNA or treated with a PKCζ inhibitor.

Wild-type mice were infected with 1x10⁹ pfu/mouse of adenovirus expressing scramble shRNA or shRNA targeting *Ppp2ca*. Mice were treated with 0.42 mg/kg of dexamethasone for 7 days. Western blotting was performed to monitor Ppp2ca protein abundance (A) and PP2A activity was measured (B) in liver. (C) Wild-type mice were treated with 0.42 mg/kg of dexamethasone for 7 days. 10 mg/kg of ACPD was injected into mice subcutaneously daily starting from day 4 for 4 days. Western blotting was performed to monitor the amount of p-PKCζ, PKCζ and Gapdh (loading control) in liver.
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Table S1. qPCR primer list.