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Chronic Glucocorticoid Exposure Induced an S1PR2-RORγ Axis to Enhance Hepatic Gluconeogenesis in Male Mice

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It is well established that chronic glucocorticoid exposure causes hyperglycemia. While glucocorticoid receptor (GR) stimulates hepatic gluconeogenic gene transcription, additional mechanisms are activated by chronic glucocorticoid exposure to enhance gluconeogenesis. We found that chronic glucocorticoid treatment activated sphingosine-1-phosphate (S1P)–mediated signaling. Hepatic knockdown of hepatic S1P receptor 1 (S1PR1) had no effect on chronic glucocorticoid-induced glucose intolerance but elevated fasting plasma insulin levels. In contrast, hepatic S1PR3 knockdown exacerbated chronic glucocorticoid-induced glucose intolerance without affecting fasting plasma insulin levels. Finally, hepatic S1PR2 knockdown attenuated chronic glucocorticoid-induced glucose intolerance and reduced fasting plasma insulin levels. Here, we focused on dissecting the role of S1PR2 signaling in chronic glucocorticoid response on glucose homeostasis. We found that chronic glucocorticoid-induced hepatic gluconeogenesis, gluconeogenic gene expression, and GR recruitment to the glucocorticoid response elements (GREs) of gluconeogenic genes were all reduced in hepatic S1PR2 knockdown male mice. Hepatic S1PR2 knockdown also enhanced glucocorticoid suppression of RAR-related orphan receptor γ (RORγ) expression. Hepatic RORγ overexpression in hepatic S1PR2 knockdown mice restored glucocorticoid-induced glucose intolerance, gluconeogenic gene expression, and GR recruitment to their GREs. Conversely, RORγ antagonist and the reduction of hepatic RORγ expression attenuated such glucocorticoid effects. Thus, chronic glucocorticoid exposure induces an S1PR2-RORγ axis to cooperate with GR to enhance hepatic gluconeogenesis. Overall, this work provides novel mechanisms of and pharmaceutical targets against steroid-induced hyperglycemia.

ARTICLE HIGHLIGHTS

- Chronic glucocorticoid exposure increased hepatic sphingosine-1-phosphate (S1P) levels in male mice, and reduced hepatic sphingosine-1-phosphate receptor 1, 2, and 3 (S1PR1–3) expression modulated glucocorticoid-regulated glucose homeostasis.
- Hepatic S1PR2 knockdown blunted chronic glucocorticoid-induced glucose intolerance and gluconeogenesis and enhanced glucocorticoid-reduced RAR-related orphan receptor γ (RORγ) expression.
- RORγ antagonist and hepatic RORγ knockdown reduced, whereas overexpressing RORγ in hepatic S1PR2 knockdown mice enhanced, glucocorticoid response on gluconeogenesis and potentiated glucocorticoid receptor occupancy at the glucocorticoid response elements of hepatic gluconeogenic genes to enhance their transcription.
- Overall, upon chronic glucocorticoid exposure, hepatic S1PR2 signaling attenuates glucocorticoid-reduced RORγ expression to enhance glucocorticoid-induced gluconeogenesis in male mice.

It is well established that chronic and excess glucocorticoid exposure, through multiple mechanisms, including prolonged

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stress, long-term glucocorticoid use for treating inflammatory and autoimmune disease, depression, chronic stress, or Cushing syndrome, cause hyperglycemia and insulin resistance (1–3). Glucocorticoids act through their intracellular glucocorticoid receptor (GR), promoting transcription of genes encoding enzymes in hepatic gluconeogenesis, such as phosphoenolpyruvate carboxykinase (*Pck1*) and the catalytic subunit of glucose 6 phosphatase (*G6pc1*) (1,4,5). Studies have shown that glucocorticoids induce additional mechanisms to enhance hepatic glucose production. We previously showed that prolonged glucocorticoid treatment increases ceramide production, a process that requires angiopoietin-like 4 (*Angptl4*), a glucocorticoid-induced gene encoding a secreted protein that inhibits lipoprotein lipase activity and promotes adipose tissue lipolysis (6–8). Ceramide-initiated signaling in the liver activates protein phosphatase 2A (PP2A) and protein kinase Cζ (PKCζ) to suppress Akt, a key component in insulin signaling, causing insulin resistance and elevating hepatic gluconeogenesis (9).

In addition to ceramides, hepatic sphingosine-1-phosphate (S1P) levels are also augmented by glucocorticoid treatment (9). Ceramides can be converted to sphingosine, which can be further metabolized to S1P. S1P is a signaling molecule whose role in glucocorticoid-mediated metabolic regulation has not been reported. S1P can exert its actions intracellularly and extracellularly (10,11). Here, we explored the potential role of extracellular S1P in chronic glucocorticoid exposure–induced glucose homeostasis disorder. Extracellular S1P binds to its cognate membrane G-protein–coupled receptors S1PR1–5 to exert its actions (12). S1PR1–3 are highly expressed in the liver (13). We found that hepatic knockdown of S1PR1, 2, and 3 in male mice all modulated glucocorticoid-regulated glucose homeostasis. Specifically, reducing hepatic S1PR2 expression blunted glucocorticoid-induced glucose intolerance and hepatic gluconeogenesis. RNA sequencing (RNA-seq) found that glucocorticoid suppression of RAR-related orphan receptor γ (*Rorc*) (called RORγ henceforward) gene expression was enhanced in glucocorticoid-treated hepatic S1PR2 knockdown mouse liver. Because RORγ has been previously shown to positively regulate hepatic gluconeogenic gene transcription (14), we further analyzed its role in chronic glucocorticoid exposure–enhanced gluconeogenesis.

RESEARCH DESIGN AND METHODS

Animals

Andras Nagy (Samuel Lunenfeld Research Institute, Mount Sinai Hospital) and Jeff Gordon (Washington University) provided *Angptl4* $^{+/+}$ and *Angptl4* $^{-/-}$ mixed C57BL/6J:129 SV background male mice, which were cohoused as previously described (15). All experiments were approved by the University of California, Berkeley (AUP-2014-07-6617). This study was performed entirely in male mice.

Adeno-Associated Virus

Adeno-associated virus serotype 8 (AAV8) expressing shRNA scramble, shRNA targeting S1PR1, S1PR2, S1PR3, RORγ, and green fluorescent protein (GFP), and mouse RORγ cDNA were purchased from Vector Biolabs (Malvern, PA) and were injected in the tail vein (3×10^{11} genome copies/mouse). The shRNA sequences are provided in Supplementary File 1.

Dexamethasone Water Supplementation

Dexamethasone (Dex) water 0.84 mg/kg body weight (PHR 1768; Sigma) was previously described (15). AAV mice were switched to Dex-supplemented water 2 days after injection for 4 weeks.

Glucose, Insulin, and Pyruvate Tolerance Tests

Mice were fasted for 16 h for intraperitoneal glucose tolerance tests (IPGTTs) (1 mg/kg D-glucose, 50-99-7; Sigma) and pyruvate tolerance tests (PTTs) (2 mg/kg sodium pyruvate, P2256; Sigma) and fasted 2 h for insulin tolerance tests (1 unit/kg of insulin, 5 mL I0516; Sigma). A Contour Next glucometer was used to measure blood glucose.

Blood and Tissue Collection

Fasted blood was collected in EDTA tubes (16.44.100; Sarstedt). CO_2 euthanasia was performed at 3–5 h into the light cycle (10:00 A.M.–12:00 P.M.). Tissues were snap frozen in liquid nitrogen and stored at −80°C.

ELISA

Plasma insulin, liver phosphorylated Akt at serine 473 (pAkt) and Akt levels, and liver and plasma S1P levels were measured using ELISA. The detailed protocols are provided in Supplementary File 1.

RNA-Seq

RNA-seq was performed by BGI Americas (Cambridge, MA). The protocol of the analysis of RNA-seq is provided in Supplementary File 1.

Western Blot

Tissues were prepared for SDS-PAGE as previously described (15). Antibody information is provided in Supplementary File 1. Images were analyzed using ImageJ software.

Gene Expression

Gene expression was performed as previously described (15), and Rpl19 was used for internal normalization. Primers are listed in Table 1.

Liver Chromatin Immunoprecipitation

Liver chromatin immunoprecipitation (ChIP) was performed as previously described (15) with the following modifications: 10 strokes with dounce homogenizer, sonication 60% amplitude (5 min total), and 10-s bursts (40-s rests). Four micrograms of the following antibodies were used: purified rabbit IgG (whole molecule) control (A01008; GeneScript), GR (IA-1 [a polyclonal rabbit antibody against human GR amino acids

Table 1—Primers

Primer name	Forward	Reverse
Quantitative PCR		
rpl19	tcc ttg gtc tta gac ctg cg	atg gag cac atc aca ag c
pck1	ctg cat aac ggt ctg gac ttc	cag caa ctg ccc gta ctc c
g6pc1	gac cat aac ata gta tac acc tgc tgc	gac cat aac ata gta tac acc tgc tgc
ChIP		
rpl19	tcc ttg gtc tta gac ctg cg	atg gag cac atc aca ag c
Pck1-GBR	gca ggc tct tgc ctt aat tg	ggt gtt ttg aca acc agc ag
G6pc1-GBR	tgc aag agt cat ggt tga aac ag	cac act gct tga cag tgc ta

84–112 QPDLSKAVSLSMGLYMGETETKVMGNDLG]; a gift from Dr. Miles Pufall, University of Iowa), and RORγ (14-6988-82; Invitrogen). Forty microliters of 25% protein A/G sepharose beads (Santa Cruz Biotechnology) were used (15).

Cell Culture and Transfection

H4IIE cells were cultured in DMEM with 10% FBS and serum starved overnight before treatment. For 4 h, 100 nmol/L Dex (D1756; Sigma), 100 nmol/L Ly3214996 (501932152; MedChemExpress), and/or 2.5 μmol/L XY018 were added. During the last 2 h, 0.5 μmol/L CYM-5520 was added (5418/10; R&D Systems). For transfection, H4IIE cells were plated at 0.5×10^6 cells/well and cultured in DMEM containing 5% FBS. The detailed protocol is provided in Supplementary File 1.

Quantification and Statistical Analysis

GraphPad Prism 9.0 software was used for the data analysis. Data are presented as mean with SD and SEM using Student *t* test, one-way ANOVA, and two-way ANOVA with Fisher least significant difference post hoc and Benjamini and Hochberg false discovery rate correction tests as indicated.

Data and Resource Availability

All data generated or analyzed during this study are included in the published article and its online supplementary files. RNA-seq data are deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (accession no. GSE150626).

RESULTS

Reducing Hepatic S1PR1–3 Signaling Modulated Glucocorticoid-Regulated Glucose Homeostasis

Wild-type (WT) male mice infected with AAV8 expressing scramble shRNA (AAV8-shScr) were treated with or without Dex (a synthetic glucocorticoid) for 4 weeks. Dex treatment resulted in glucose intolerance (Fig. 1*A*). As previously reported, hepatic, but not plasma, S1P levels were elevated by Dex treatment (9) (Fig. 1*B*). To learn whether extracellular S1P is involved in glucocorticoid-regulated glucose homeostasis, WT mice were infected with AAV8 expressing shRNA targeting S1PR1 (AAV8-shS1PR1), AAV8-shS1PR2, or AAV8-shS1PR3. These mice, along with AAV8-shScr mice, were treated with Dex for 4 weeks. The expression of S1PR1, 2, and 3 was reduced in the liver but not in gastrocnemius muscle, inguinal white adipose tissue, or brown adipose tissue (Fig. 1*C* and Supplementary Fig. 1*A*). Hepatic S1PR1 knockdown had no effect, whereas hepatic S1PR3 knockdown further exacerbated Dex-induced glucose intolerance (Fig. 1*D*). In contrast, hepatic S1PR2 knockdown blunted the Dex response (Fig. 1*D*). Similarly, hepatic S1PR1 knockdown had no effect on Dex-induced fasting hyperglycemia, while hepatic S1PR3 knockdown worsened and hepatic S1PR2 knockdown improved Dex-induced fasting hyperglycemia (Fig. 1*E*). Compared with AAV8-shScr mice, fasting plasma insulin levels were lower in AAV8-shS1PR2 mice and higher in AAV8-shS1PR1 mice, but fasting insulin levels were not different between AAV8-shS1PR3 and AAV8-shScr mice (Fig. 1*F*). Overall, these results demonstrate that reducing hepatic S1PR1, 2, and 3 expression all affected glucose homeostasis in the presence of Dex. Importantly, reducing their hepatic expression did not affect plasma ALT levels (Supplementary Fig. 1*B*). Thus, hepatic S1PR1, 2, and 3 knockdowns did not cause liver injury. Of note, we found that without Dex treatment, AAV8-shS1PR2 mice were also more glucose tolerant than AAV8-shScr mice (Fig. 1*G*), though there were no differences in fasting plasma insulin levels (Fig. 1*H*). Next, we specifically investigated the mechanism underlying the effects of altered hepatic S1PR2 signaling in the context of chronic glucocorticoid exposure–induced glucose disorder.

Because hepatic S1P induction by Dex depends on the presence of ANGPTL4 in male mice (9), we tested whether the effects of S1PR2 knockdown on glucose tolerance also requires ANGPTL4. As previously reported, Dex-induced glucose intolerance was compromised in *Angptl4*$^{-/-}$ mice (9) (Supplementary Fig. 2). In contrast to the observations in WT mice, hepatic S1PR2 knockdown did not affect glucose tolerance in Dex-treated *Angptl4*$^{-/-}$ mice (Supplementary Fig. 2), and a trend toward improved glucose tolerance was observed in *Angptl4*$^{-/-}$ mice not treated with Dex (Supplementary Fig. 2). These results demonstrate that while ANGPTL4 is required for the effects of S1PR2 signaling in the context of chronic Dex treatment–induced glucose intolerance, it perhaps is not required for the effects of S1PR2 signaling with respect to the regulation of basal glucose tolerance.

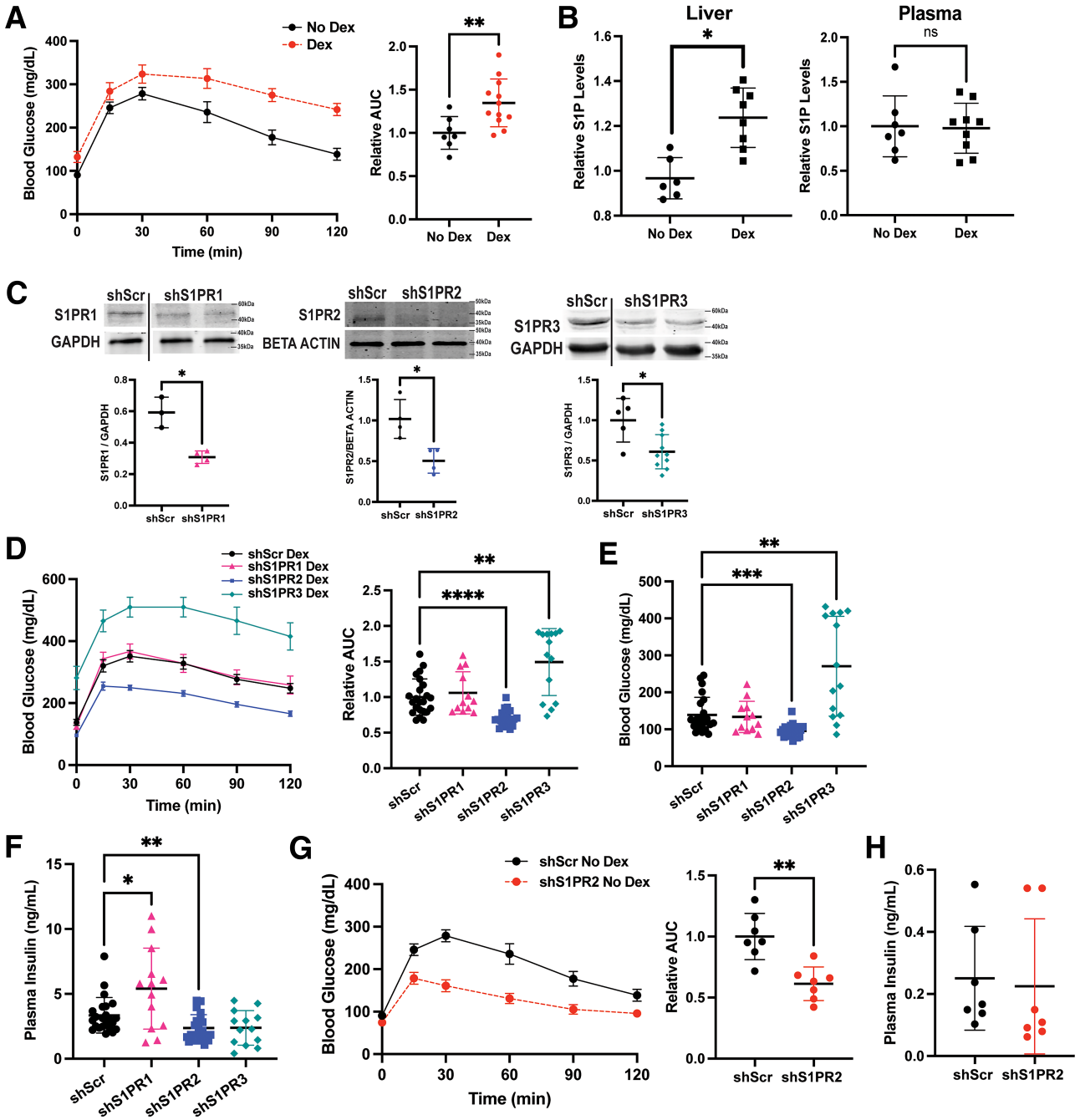


Figure 1—Hepatic S1PR2 knockdown but not S1PR1 and S1PR3 improved chronic glucocorticoid exposure–induced glucose intolerance. *A*: IPGTT and relative area under the curve (AUC) of WT male AAV8-shScr mice treated without Dex and with Dex (0.84 mg/kg body weight) for 4 weeks after a 16-h fast (n = 7–12). $^{**}P < 0.005$ using Student t test with Welch correction. Error bars represent SD and SEM for the tolerance test. *B*: Hepatic and plasma S1P levels in WT male mice undergoing Dex treatment (4 weeks) (n = 6–10). *C*: Western blots of S1PR1, S1PR2, and S1PR3 in AAV8-shScr, AAV8-shS1PR1, AAV8-shS1PR2, and AAV8-shS1PR3 male mouse liver. Images are from the same blot, and groups were cropped for ease of visualization (n = 3–9). *D*: IPGTT and relative AUC of AAV8-shScr, AAV8-shS1PR1, AAV8-shS1PR2, and AAV8-shS1PR3 mice treated with Dex (0.84 mg/kg body weight) for 4 weeks after 16-h fast (n = 14–23). *E*: Fasting blood glucose levels of AAV8-shScr, AAV8-shS1PR1, AAV8-shS1PR2, and AAV8-shS1PR3 mice treated with Dex after 16-h fast. *F*: Plasma insulin of AAV8-shScr, AAV8-shS1PR1, AAV8-shS1PR2, and AAV8-shS1PR3 mice treated with Dex after 16-h fast (n = 7–13). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ using ANOVA with Welch correction. Error bars represent SEM for the tolerance test and SD for the rest. *G*: IPGTT and relative AUC of AAV8-shScr and AAV8-shS1PR2 without Dex (n = 7). *H*: Plasma insulin of AAV8-shScr and AAV8-shS1PR2 mice without Dex after 16-h fast (n = 7). $^{**}P < 0.005$ using Student t test with Welch correction. Error bars represent SEM for the tolerance test and SD for the rest.

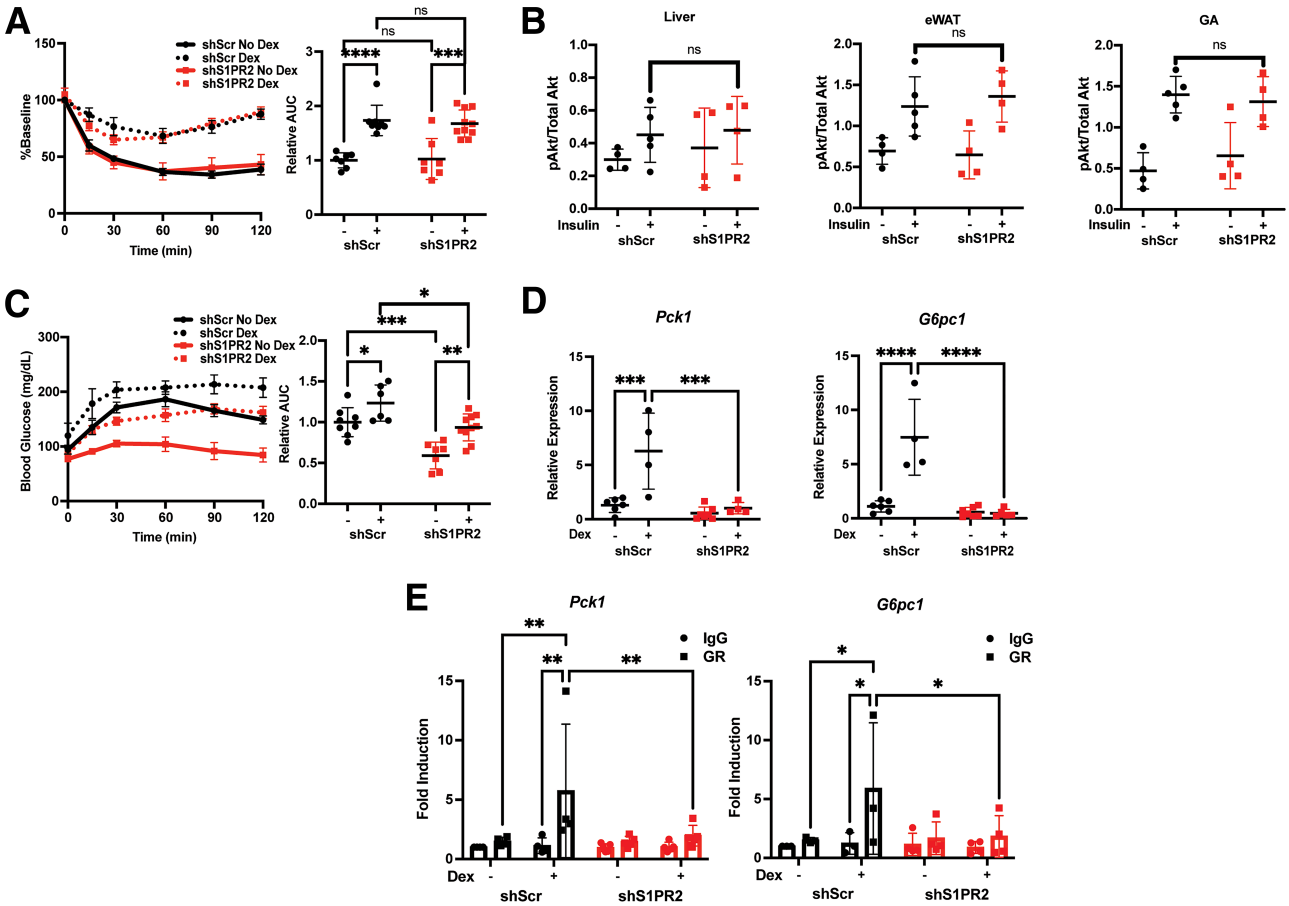


Figure 2—Hepatic S1PR2 knockdown reduced Dex-induced pyruvate tolerance but not insulin intolerance. *A*: Insulin tolerance test of AAV8-shScr and AAV8-shS1PR2 male mice treated with or without Dex for 4 weeks after 2-h fast (n = 6–12). *B*: pAkt/Akt ratio determined through ELISA of the liver, epididymal white adipose tissue (eWAT), and gastrocnemius muscle (GA) in AAV8-shScr and AAV8-shS1PR2 mice treated with Dex and injected with or without insulin (n = 4–5). *C*: PTT in AAV8-shScr and AAV8-shS1PR2 mice treated with or without Dex after 16-h fast (n = 6–10). *D*: Gene expression in AAV8-shScr and AAV8-shS1PR2 mice treated with or without Dex (n = 4–5). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ using two-way ANOVA with Benjamini and Hochberg false discovery rate correction. Error bars represent the SD. *E*: GR ChIP on *Pck1* and *G6pc1* (n = 3–5). $^*P < 0.05$, $^{**}P < 0.01$, using a two-way ANOVA with Fisher least significant difference post hoc test. Error bars represent SEM for the tolerance tests and SD for the rest. AUC, area under the curve.

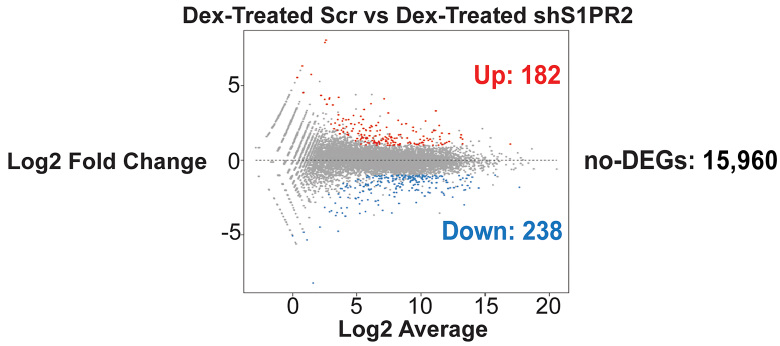
Hepatic S1PR2 Knockdown Did Not Affect Insulin Tolerance but Reduced Gluconeogenesis in Dex-Treated Mice

We performed an insulin tolerance test on AAV8-shScr and AAV8-shS1PR2 mice treated with or without Dex. Without Dex, these mice had similar insulin tolerance (Fig. 2*A*). Dex treatment induced insulin intolerance, but, interestingly, hepatic S1PR2 knockdown did not affect Dex-induced insulin intolerance (Fig. 2*A*). This observation is consistent with analyses of the ratio of pAkt and total Akt (pAkt/Akt), an indicator of insulin signaling activity (16) in liver, gastrocnemius muscle, and epididymal white adipose tissue in AAV8-shScr and AAV8-shS1PR2 mice treated with Dex and injected with insulin or PBS. pAkt/Akt levels were similar in all three tissues examined between AAV8-shScr and AAV8-shS1PR2 mice (Fig. 2*B*). Thus, reducing hepatic S1PR2 signaling did not affect Dex-modulated insulin signaling in these tissues.

We next performed a PTT to monitor the hepatic gluconeogenic capacity of AAV8-shScr and AAV8-shS1PR2 mice treated with or without Dex. Dex treatment increased fasting glucose levels and gluconeogenesis in AAV8-shScr mice (Fig. 2*C*). Hepatic S1PR2 knockdown reduced gluconeogenic capacity in both control and Dex-treated mice (Fig. 2*C*). Gene expression analysis revealed that hepatic S1PR2 knockdown reduced the expression of gluconeogenic genes *Pck1* and *G6pc1* (Fig. 2*D*). Notably, without Dex, *Pck1* and *G6pc1* expressions were similar between these mice (Fig. 2*D*). These indicate that S1PR2 signaling is involved in Dex-stimulated and basal gluconeogenesis. However, the mechanisms underlying S1PR2's role in these two processes are different.

One potential mechanism to explain the lower induction of gluconeogenic genes by Dex in hepatic S1PR2 knockdown mouse liver would be through reduced Dex-induced recruitment of GR to the GREs of gluconeogenic genes. We performed GR ChIP to examine this model. Without Dex, GR occupancy on the GREs was minimal in AAV8-shScr and AAV8-shS1PR2 mouse liver (Fig. 2*E*). Dex treatment significantly increased GR recruitment to the GREs of *Pck1*

A



B

Genes induced by hepatic S1PR2 knockdown

Annotation Category	P value
Immune Response	8.76E-08
Defense to Virus	1.69E-06
Response to Estradiol	2.39E-06
Lipid Transport	4.59E-05
Lipoprotein Metabolic Process	1.51E-04

Genes reduced by hepatic S1PR2 knockdown

Annotation Category	P value
Epoxygenase P450 Pathway	2.70E0-8
Lipid Metabolic Process	6.92E-07
Steroid Metabolic Process	4.32E-05
Acetyl-CoA Metabolic Process	3.65E-05
Glycolytic Process via Fructose-1-phosphate	3.52E-04

C

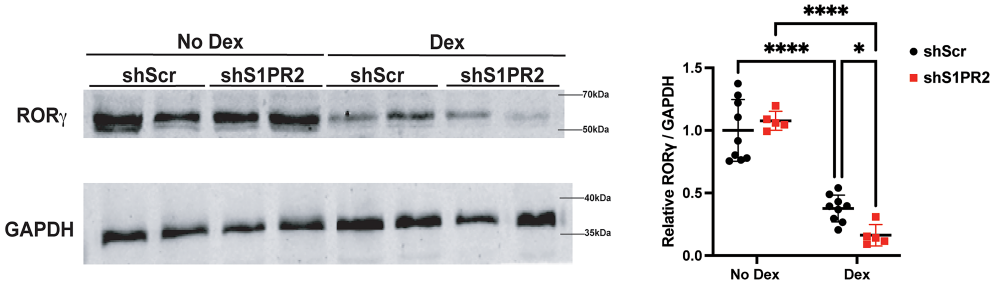


Figure 3—Hepatic knockdown of S1PR2 reduces RORγ expression. *A*: MA plot of RNA-seq analysis of genes in the liver of Dex-treated AAV8-shScr and Dex-treated AAV8-shS1PR2 male mice with twofold difference and adjusted *P* value of 0.05 as the cutoff. *B*: Gene ontology analysis of hepatic genes induced and reduced by hepatic S1PR2 knockdown. *C*: Western blot for RORγ in AAV8-shScr and AAV8-shS1PR2 mice treated with and without Dex. Relative expression was normalized to GAPDH using ImageJ software ($n = 3$). *$P < 0.05$, ****$P < 0.0001$ using two-way ANOVA with Fisher least significant difference post hoc test. Error bars represent the SD. no-DEGs, number of differentially expressed genes.

(4) and *G6pc1* (17) genes in AAV8-shScr mouse liver (Fig. 2*E*). However, GR recruitment was significantly lower in Dex-treated AAV-shS1PR2 mice than Dex-treated AAV8-shScr mice (Fig. 2*E*).

Hepatic S1PR2 Knockdown Reduced the Expression of RORγ

To probe potential mechanisms for the effects of S1PR2 knockdown, we performed RNA-seq to identify differentially expressed genes in Dex-treated AAV8-shScr versus Dex-treated AAV8-shS1PR2 mouse liver. Using a twofold difference and adjusted *P* value of 0.05 as the cutoff, we found that 238 genes had lower expression, and 182 genes were higher in Dex-treated AAV8-shS1PR2 versus Dex-treated AAV8-shScr mouse liver (Fig. 3*A*). A list of these genes is presented in Supplementary File 2. Gluconeogenic genes were among the list of downregulated genes in AAV8-shS1PR2 liver, including *G6pc1*, fructose bisphosphatase 1 (*Fbp1*), and tyrosine aminotransferase (*Tat*). *Pck1* was not scored based on the cutoff standard, but conventional real-time PCR analysis clearly showed a lower *Pck1* expression in the liver of Dex-treated AAV8-shS1PR2 mice (Fig. 2*D*). Moreover, RNA-seq also found that S1PR2 knockdown reduced the expression of *Rorc*, which encodes a nuclear factor that can potentiate *Pck1* and *G6pc1* transcription (14). Gene ontology analysis found that genes encoding the epoxygenase p450 pathway, lipid, steroid, acetyl-CoA, and glycolytic metabolic processes were highly represented among S1PR2 knockdown–reduced genes (Fig. 3*B*). In contrast, genes involved in immune response, antiviral defense, estrogen responses, lipid transport, and lipoprotein metabolism were highly represented among S1PR2 knockdown–induced genes (Fig. 3*B*). Thus, S1PR2 signaling participates in many facets of glucocorticoid actions. The role of RORγ in glucocorticoid-regulated hepatic gluconeo-

genesis was of particular interest since this had not been previously reported. Immunoblotting showed that without Dex, S1PR2 knockdown did not affect RORγ expression (Fig. 3*C*). While Dex treatment reduced RORγ expression in the liver AAV8-shScr mice, Dex suppression of RORγ expression was even more pronounced in the liver of AAV8-shS1PR2 mice (Fig. 3*C*). Similarly, nuclear levels of RORγ were suppressed in Dex-treated AAV8-shScr and further suppressed in AAV8-shS1PR2 mice (Supplementary Fig. 3*A*). RORα has also been shown to activate the *Pck1* gene (18,19). However, protein levels of RORα were not affected by hepatic S1PR2 knockdown (Supplementary Fig. 3*B*).

RORγ Was Involved in Dex-Induced Gluconeogenic Gene Transcription

To explore whether RORγ is involved in Dex-regulated gluconeogenic gene expression, H4IIE rat hepatoma cells were treated with *1*) DMSO (vehicle), *2*) Dex, *3*) CYM-5520 (an agonist of S1PR2) (20), *4*) Dex plus CYM-5520, *5*) XY018 (an RORγ antagonist) (21), *6*) Dex plus XY108, *7*) CYM-5520 plus XY018, and *8*) Dex plus CYM-5520 plus XY108 for 4 h, then *Pck1* and *G6pc1* expression were analyzed. *Pck1* and *G6pc1* expression were induced by Dex but not by CYM-5520 (Fig. 4*A*). However, CYM-5520 enhanced Dex-induced *Pck1* and *G6pc1* expression (Fig. 4*A*). XY018 did not affect basal *Pck1* and *G6pc1* expression but suppressed both Dex and Dex plus CYM-5520–induced *Pck1* and *G6pc1* expression (Fig. 4*A*). Thus, RORγ activity was required for maximal Dex and S1PR2 signaling response on gluconeogenic gene expression.

RORγ Antagonist Improved Dex-Induced Glucose Intolerance and Hepatic Gluconeogenesis

We next tested RORγ's role in Dex-induced glucose intolerance in vivo. WT male mice were treated with or without Dex for 4 weeks. For the final 2 weeks, one-half of each group was treated with PBS or XY018. Dex-treated mice with PBS injections were more glucose intolerant than mice that only received PBS injections (Fig. 4*B*). XY018 treatment alleviated Dex-induced glucose intolerance but did not affect basal glucose tolerance (Fig. 4*B*). PTTs showed that Dex treatment augmented glucose production in WT mice, whereas XY018 alone had no effect (Fig. 4*C*). Dex-treated mice that also received XY018 had a lower gluconeogenic capacity than mice treated with Dex alone (Fig. 4*C*). Not surprisingly, XY018 reduced *Pck1* and *G6pc1* expression in Dex-treated WT mice (Fig. 4*D*). XY018 did not affect *Pck1* and *G6pc1* gene expression in mice not treated with Dex (Fig. 4*D*). These results were consistent with the results observed in H4IIE cells.

We performed liver ChIP in these mice to monitor the GR and RORγ occupancy at the RORγ response elements (ROREs) (18) and GREs (4) of *Pck1* and *G6pc1* (Fig. 4*E*). Without treatment, GR was modestly recruited to the GREs (Fig. 4*E*). This occupancy was likely due to endogenous corticosterone. RORγ, however, was not found at the ROREs (Fig. 4*E*). Dex treatment increased both GR and RORγ recruitment to the GRE-RORE regions of these two genes (Fig. 4*E*). XY018 did not affect basal GR and RORγ occupancy but attenuated Dex-induced GR and RORγ recruitment (Fig. 4*E*).

Hepatic RORγ Knockdown Improved Dex-Induced Glucose Intolerance and Reduced Gluconeogenesis

WT mice were infected with AAV8-shScr or shRNA targeting *Rorc* (AAV8-shRORγ) and treated with Dex. Immunoblotting confirmed a significantly reduced RORγ expression in the liver, but not brown adipose tissue or gastrocnemius muscle, in AAV8-shRORγ mice (Fig. 5*A*). Dex-treated AAV8-shRORγ mice showed a trend toward better glucose tolerance than AAV8-shScr mice (Fig. 5*B*). Moreover, PTT showed that hepatic gluconeogenesis was significantly lower in Dex-treated AAV8-shRORγ mice (Fig. 5*C*). Fasting plasma insulin levels were also lower in AAV8-shRORγ mice (Fig. 5*D*). Interestingly, while *Pck1* expression was reduced in livers of AAV8-shRORγ mice, there were no differences in *G6pc1* expression (Fig. 5*E*). Nonetheless, the results from hepatic RORγ knockdown mice generally agreed with the observation from the RORγ antagonist studies in Fig. 4. Notably, hepatic RORγ knockdown did not affect plasma ALT levels (Supplementary Fig. 4*B*).

To further test the functional interaction between RORγ and GR, a rat *Pck1* promoter luciferase reporter was transfected into H4IIE cells along with expression plasmids of human GR and human RORγ (hRORγ). Without hRORγ overexpression, Dex treatment induced the reporter activity (Fig. 5*F*). hRORγ overexpression did not affect the reporter activity. However, transfecting 300 ng of hRORγ expression plasmid significantly potentiated the Dex response (Fig. 5*F*). We tested whether an RORγ agonist can further potentiate this response. In separate experiments, H4IIE cells were transfected with a rat *Pck1* promoter reporter, expression plasmids of human GR, and 300 ng hRORγ. Twenty-four hours after transfection, cells were treated with DMSO, Dex, LYC-55716 (an RORγ agonist) (22), and Dex plus LYC-55716. Interestingly, 100 nmol/L LYC-55716 treatment alone showed a trend toward increased basal reporter activity. Both 50 nmol/L and 100 nmol/L LYC-55716 significantly enhanced the Dex response (Fig. 5*G*). Overall, these results suggest that RORγ enhances GR activation of rat *Pck1* promoter.

Hepatic RORγ Overexpression Restored Glucose Intolerance and Gluconeogenesis Induced by Dex Treatment in AAV8-shS1PR2 Mice

Next, we tested whether RORγ overexpression in hepatic S1PR2 knockdown mice restored Dex-induced glucose intolerance and gluconeogenesis. Male WT mice infected with AAV8-shS1PR2 were treated with or without Dex. One week later, they were infected with AAV8 expressing GFP (AAV8-shS1PR2-GFP) or mouse RORγ (AAV8-shS1PR2-RORγ). Dex treatment was continued for 3 more weeks, and IPGTT and PTT were performed. Western blotting validated liver specificity of RORγ overexpression (Fig. 6*A*). Dex

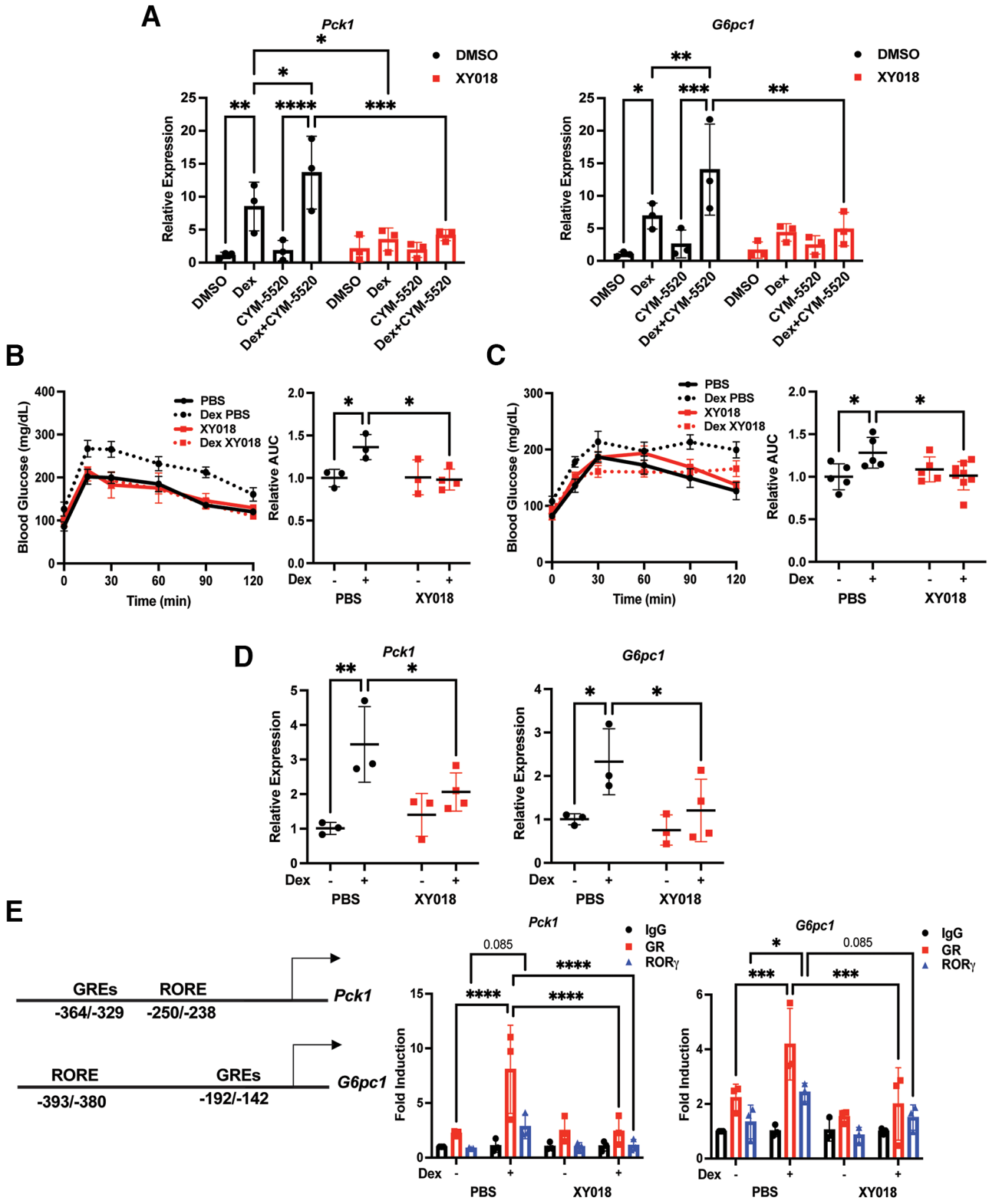


Figure 4—RORγ antagonist reduces Dex-activated hepatic gluconeogenesis. *A*: RORγ antagonist (XY018) reduces Dex-induced gluconeogenic gene expression. These experiments were repeated three times, and each time had triplicates: 100 nmol/L Dex and/or 0.5 μmol/L S1PR2 agonist CYM-5520 and/or 2.5 μmol/L RORγ antagonist (XY018). *B*: XY018 5 mg/kg was intraperitoneally injected daily in male mice for the last 2 weeks of the 4-week Dex treatment and an IPGTT was done after a 16-h fast (n = 3–4). *C*: XY018 5 mg/kg was intraperitoneally injected twice per every 3 days of the last 2 weeks of the 4-week Dex treatment, and a PTT was done after a 16-h fast (n = 5–8). *D*: Hepatic gluconeogenic gene expression WT mice treated without Dex (−) or with 0.84 mg/kg body weight Dex (+) and with PBS or 5 mg/kg RORγ antagonist (XY018) (n = 3–4). *E*: The location of mouse *Pck1* and *G6pc1* GREs and ROREs and ChIP of GR and RORγ on the GRE-RORE region of mouse *Pck1* and *G6pc1*

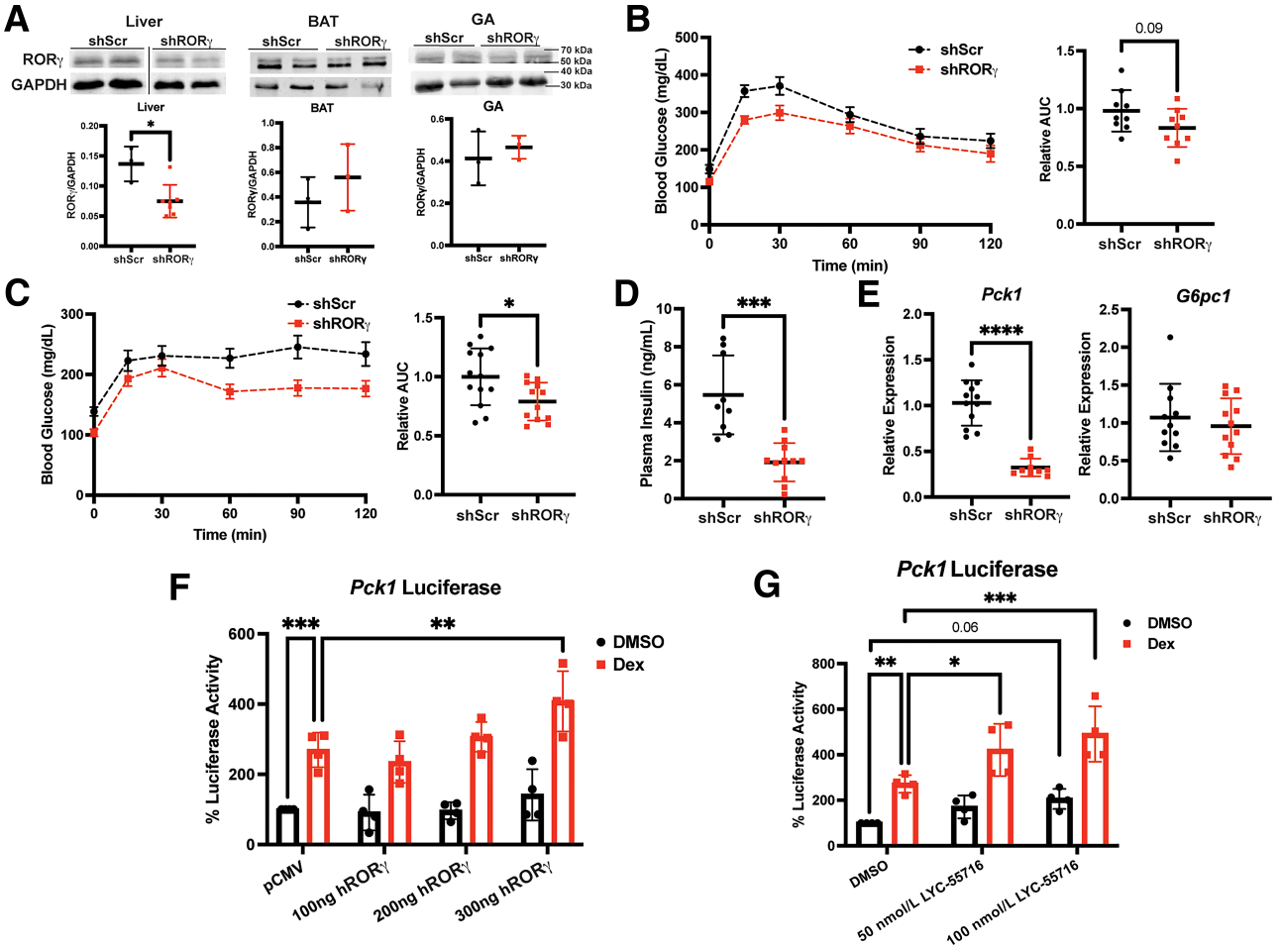


Figure 5—Hepatic RORγ knockdown reduced Dex-induced gluconeogenesis in male mice. *A*: Western blots of RORγ in AAV8-shScr and AAV8-shRORγ liver, brown adipose tissue (BAT), and gastrocnemius muscle (GA). Relative expression normalized to GAPDH using ImageJ software (n = 3–7). *B*: IPGTT and relative area under the curve (AUC) of AAV8-shScr and AAV8-shRORγ mice treated with Dex after a 16-h fast (n = 9). *C*: PTT and relative AUC of AAV8-shScr and AAV8-shRORγ mice treated with Dex after a 16-h fast (n = 12–15). *D*: Plasma insulin levels of AAV8-shScr and AAV8-shRORγ mice treated with Dex after a 16-h fast (n = 11–13). *E*: Hepatic gluconeogenic gene expression AAV8-shScr and AAV8-shRORγ mice treated with Dex (n = 6–8). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ by Student t test with Welch correction. The error bars represent the SD. *F*: Rat *Pck1* promoter luciferase activity in H4IIE cells transfected with porcine cytomegalovirus (pCMV) (control), and 100 ng, 200 ng, or 300 ng hRORγ treated with or without 500 nmol/L Dex (n = 4). *G*: Rat *Pck1* promoter luciferase activity in H4IIE cells transfected with 300 ng hRORγ, treated with or without 500 nmol/L Dex, and treated with DMSO, 50 nmol/L, or 100 nmol/L RORγ agonist (LYC-55716) (n = 4). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by two-way ANOVA with Fisher least significant difference post hoc test. Error bars represent SEM for the tolerance tests and SD for the rest.

treatment caused glucose intolerance and increased gluconeogenesis in AAV8-shS1PR2-GFP mice (Fig. 6*B* and *C*). Overexpression of RORγ further enhanced these phenotypes (Fig. 6*B* and *C*). Hepatic *Pck1* and *G6pc1* expression was also higher in Dex-treated AAV8-shS1PR2-RORγ mice (Fig. 6*D*). Without Dex, RORγ overexpression did not increase glucose intolerance or gluconeogenesis of AAV8-shS1PR2 mice (Fig. 6*B* and *C*). Thus, RORγ is not involved in the regulation of basal glucose tolerance and gluconeogenesis regulated by S1PR2 signaling.

The results of ChIP assays showed that the Dex effect on GR recruitment to the *Pck1* and the *G6pc1* GREs in AAV8-shS1PR2-GFP mice was minimal (Fig. 6*E*), similar to the observation in AAV8-shS1PR2 mice (Fig. 2*E*). Overexpression of RORγ enhanced GR recruitment (Fig. 6*E*). RORγ was not present in the *Pck1* and *G6pc1* ROREs in AAV8-shS1PR2-GFP mice (Fig. 6*E*). Surprisingly, without Dex, RORγ was also not present in the *Pck1* and *G6pc1* ROREs in AAV8-shS1PR2-RORγ mouse liver (Fig. 6*E*). RORγ was only recruited to the ROREs in AAV8-shS1PR2-RORγ mouse liver upon Dex treatment (Fig. 6*E*).

genes of liver of WT mice treated without Dex (−) or with Dex (+) and with PBS or 5 mg/kg RORγ antagonist (XY018) (n = 3–4). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ by two-way ANOVA with Fisher least significant difference post hoc test. Error bars represent SEM for the tolerance tests and SD for the rest. AUC, area under the curve.

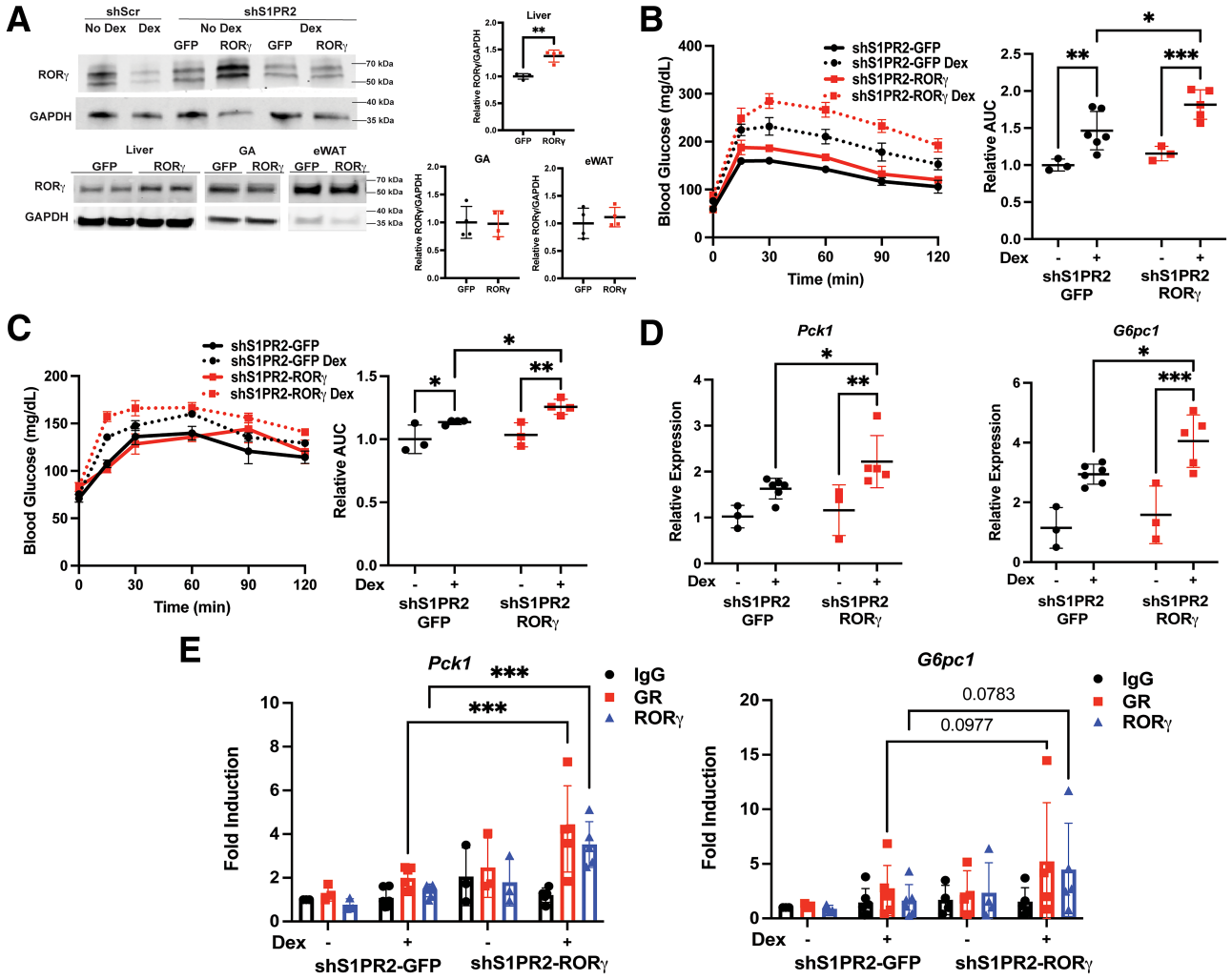


Figure 6—RORγ overexpression in AAV8-shS1PR2 male mice worsens 4 weeks Dex treatment–induced glucose and pyruvate tolerance. *A*: RORγ protein expression by Western blot in liver, gastrocnemius muscle (GA), and epididymal white adipose tissue (eWAT) (n = 4). $^{**}P < 0.01$ by Student t test. *B*: IPGTT after a 16-h fast. *C*: PTT after a 16-h fast. *D*: Hepatic gluconeogenic gene expression. *E*: ChIP of GR and RORγ on the GRE-RORE region of mouse *Pck1* and *G6pc1* genes of the liver of AAV8-shS1PR2 mice overexpressing IgG, GR, or RORγ and treated without Dex (−) or with Dex (+) (n = 3–6). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, using two-way ANOVA with Fisher least significant difference post hoc test. Error bars represent SEM for the tolerance tests and SD for the rest.

Overall, these results demonstrate that upon chronic Dex treatment, RORγ is recruited to the ROREs of *Pck1* and *G6pc1* genes and is required for maximal GR recruitment to the GREs. This augments the expression of hepatic gluconeogenic genes, which enhances gluconeogenesis and glucose intolerance (Fig. 7).

DISCUSSION

In this study, we showed that chronic Dex treatment increased liver S1P levels in male mice and that reducing the expression of hepatic S1PR1, 2, and 3 all modulated the effect of Dex treatment on glucose metabolism, though with distinct effects. Hepatic S1PR3 knockdown exacerbated Dex-induced glucose intolerance and fasting hyperglycemia but had no effect on fasting hyperinsulinemia. In contrast, hepatic S1PR1 knockdown did not affect glucose tolerance and fasting glucose levels but increased fasting insulin levels. We speculate that S1PR3 signaling negatively regulates chronic glucocorticoid-induced hepatic gluconeogenesis, whereas S1PR1 signaling attenuates chronic glucocorticoid-induced insulin resistance. These models will be examined in our future studies. We also found that hepatic S1PR2 knockdown attenuated Dex-induced glucose intolerance. However, hepatic S1PR2 knockdown had no effect on insulin tolerance, and Dex effect on insulin stimulated Akt activity in the metabolic tissues tested. PTTs showed that S1PR2 knockdown reduced Dex-induced gluconeogenesis. Therefore, prolonged Dex treatment induced hepatic S1PR2 signaling to enhance gluconeogenesis without affecting insulin signaling. This report focuses on elucidating the role of hepatic S1PR2 in chronic glucocorticoid-induced glucose disorders.

RNA-seq identified *Rorc* as a gene whose expression was reduced in the liver by hepatic S1PR2 knockdown in

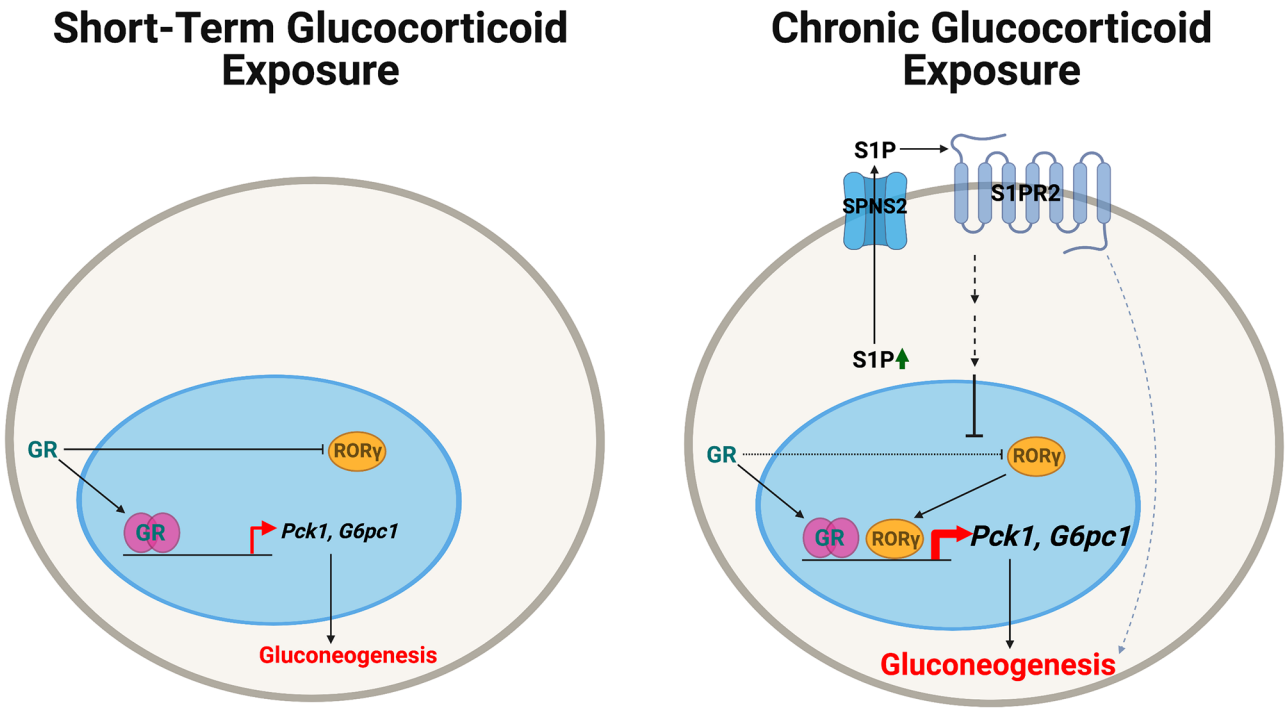


Figure 7—Model of chronic glucocorticoid exposure–induced S1PR2-RORγ axis on gluconeogenic gene expression regulation. Short-term glucocorticoid exposure, such as 6 h or an overnight fast, induces hepatic glucose production through GR binding to GREs of gluconeogenic genes while suppressing RORγ expression. Chronic glucocorticoid exposure increases hepatic production of S1P, which can be exported extracellularly where S1P binds to S1PR2 on its own cell surface or neighboring cells. S1PR2-initiated signaling antagonizes glucocorticoid-suppressed RORγ expression. The increased RORγ then enhances gluconeogenic gene transcription, as RORγ can potentiate GR recruitment to GREs of gluconeogenic genes. Hepatic S1PR2 signaling also reduces basal gluconeogenesis by unknown mechanisms.

Dex-treated mice. RORγ was previously shown to potentiate transcription of gluconeogenic genes (14), but its role in glucocorticoid's effects has not been explored. We found that RORγ overexpression in hepatic S1PR2 knockdown mouse liver restored Dex-induced glucose intolerance and increased gluconeogenesis and gluconeogenic gene expression. In contrast, RORγ antagonist XY018 treatment attenuated Dex-induced glucose intolerance and reduced gluconeogenic gene expression and hepatic gluconeogenesis. Similarly, Dex-induced hepatic gluconeogenesis was lower in hepatic RORγ knockdown mice, and they were trending toward having better glucose tolerance under Dex treatment. These results demonstrated RORγ's participation in chronic Dex-induced hepatic gluconeogenesis. RORγ overexpression in mouse liver did not affect *Pck1* and *G6pc1* basal expression or basal glucose and pyruvate tolerance. Similarly, XY018 did not affect basal glucose tolerance or hepatic gluconeogenesis. The studies of *Pck1* and *G6pc1* mRNA in H4IIE cells were consistent with these in vivo results. A previous report showed that liver-specific *Rorc* knockout mice had lower gluconeogenic activity and gluconeogenic gene expression during the daytime (14). Our results did not exactly contradict these results. Perhaps the dose and/or duration of XY018 we administered was sufficient to improve Dex-induced glucose intolerance but not enough to affect basal glucose tolerance in control mice. In fact, in rat *Pck1* promoter reporter assay, we found that RORγ agonist LYC-55176 was trending toward increasing the reporter gene activity. Interestingly, reporter gene studies showed that even without adding LYC-55716, overexpressing RORγ was able to potentiate Dex response, and LYC-55176 further enhanced this effect. This suggests that endogenous ligands of RORγ are enough to potentiate the Dex response to a certain degree and that more agonists further enhanced the effect.

In any case, because hepatic S1PR2 knockdown improved basal glucose tolerance and reduced basal gluconeogenesis, we propose that S1PR2 signaling exerts at least two mechanisms that enhance chronic glucocorticoid exposure–induced hepatic gluconeogenesis. The first mechanism is through suppressing glucocorticoid-reduced RORγ expression. The other is to regulate basal gluconeogenesis, for which the mechanisms remain unclear. Although S1PR2 signaling did not regulate basal gluconeogenic gene expression, other mechanisms could modulate the rate of gluconeogenesis (23). Notably, the regulation of basal glucose tolerance by S1PR2 signaling was likely independent of the presence of ANGPTL4. In contrast, ANGPTL4 was required for S1PR2 signaling to enhance glucocorticoid-induced glucose intolerance. This is not surprising because ANGPTL4 was required for Dex-induced hepatic S1P production (9).

A previous study showed that basal glucose tolerance was not different between WT and *S1pr2* null mice fed a chow diet (24). Here, we specifically reduced S1PR2 in the liver of male mice. Perhaps S1PR2 signaling in different tissues has distinct roles on whole-body glucose homeostasis. In *S1pr2*-null mice, S1PR2 signaling in certain tissues somehow offsets its role in liver gluconeogenesis.

The recruitment of GR to *Pck1* and *G6pc1* GREs was decreased in hepatic S1PR2 knockdown mouse liver. When RORγ was overexpressed in the liver of these mice, it restored GR occupancy. In contrast, XY018 attenuated Dex-induced GR occupancy. Thus, mechanistically, RORγ assists GR recruitment to the GREs of gluconeogenic genes. Previous studies of glucocorticoid activation of rat *Pck1* gene found that HNF4 and FoxA1/2 (also known as HNF3α/β) bind upstream of GREs (25,26) and enhance GR binding to the GREs (27). The RORE in the mouse *Pck1* gene overlaps with another accessory element required for maximal glucocorticoid response located downstream of GREs that has been previously shown to bind to chicken ovalbumin upstream promoter transcription factor (COUP-TF) (28). The fact that XY018 reduced GR and RORγ recruitment to the GRE-ROREs of gluconeogenic genes suggests that RORγ's transcriptional activation activity is required for its enhancement of Dex response. This is reminiscent of the requirement of HNF4 and FoxA's transactivation domains to assist GR with activating rat *Pck1* gene transcription (29). RORγ likely participates in Dex-stimulated gluconeogenic gene transcription through recruiting transcriptional coactivators into a GR-containing transcriptional complex.

Overall, the novel GR-SIPR2-RORγ axis identified here provides valuable insights to improve glucocorticoid therapy. However, two important questions remain to be explored. First, glucocorticoids are potent anti-inflammatory and immunomodulatory agents, but their applications are limited by their adverse effects, including hyperglycemia. This study suggests that including the antagonists (ideally liver specific) of S1PR2 and/or RORγ in glucocorticoid therapy to dampen hyperglycemia could provide improved flexibility of glucocorticoid use. Notably, there are active studies ongoing for designing antagonists of both S1PR2 (10,30) and RORγ (31,32) for other diseases. Obviously, this approach requires that S1PR2 and/or RORγ antagonists do not affect the anti-inflammatory response of glucocorticoids. This notion should be confirmed. Second, how hepatic S1PR2 signaling antagonizes glucocorticoid-suppressed RORγ expression is unclear. This mechanism should be explored in future studies.

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Author Contributions. R.A.L. and M.C. analyzed the data. R.A.L., M.C., A.T., Y.R.L., D.L., N.Y., S.T., and M.Z. executed the experiments. R.A.L., M.C., R.M.O., and J.-C.W. wrote the manuscript. R.A.L., M.C., and J.-C.W designed and supervised the experiments. R.A.L and J.-C.W. planned and conceptualized the study. J.-C.W. is the guarantor of this work and, as such, had full access to all data in the study and takes responsibility for the integrity of the data and accuracy of data analysis.

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