Separating the Anti-Inflammatory and Diabetogenic Effects of Glucocorticoids Through LXRβ Antagonism

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Synthetic glucocorticoids (GCs), including dexamethasone (DEX), are powerful anti-inflammatory drugs. Long-term use of GCs, however, can result in metabolic side effects such as hyperglycemia, hepatosteatosis, and insulin resistance. The GC receptor (GR) and liver X receptors (LXRα and LXRβ) regulate overlapping genes involved in gluconeogenesis and inflammation. We have previously shown that *Lxr*$\beta^{-/-}$ mice are resistant to the diabetogenic effects of DEX but still sensitive to its immunosuppressive actions. To determine whether this finding could be exploited for therapeutic intervention, we treated mice with GSK2033, a pan-LXR antagonist, alone or combined with DEX. GSK2033 suppressed GC-induced gluconeogenic gene expression without affecting immune-responsive GR target genes. The suppressive effect of GSK2033 on DEX-induced gluconeogenic genes was specific to LXRβ, was liver cell autonomous, and occurred in a target gene-specific manner. Compared with DEX treatment alone, the coadministration of GSK2033 with DEX decreased the recruitment of GR and its accessory factors MED1 and C/EBPβ to the phosphoenolpyruvate carboxykinase promoter. However, GSK2033 had no effect on DEX-mediated suppression of inflammatory genes expressed in the liver or in mouse primary macrophages stimulated with lipopolysaccharides. In conclusion, our study provides evidence that the gluconeogenic and immunosuppressive actions of GR activation can be mechanistically dissociated by pharmacological antagonism of LXRβ. Treatment with an LXRβ antagonist could allow the safer use of existing GC drugs in patients requiring chronic dosing of anti-inflammatory agents for the treatment of diseases such as rheumatoid arthritis and inflammatory bowel disease. (***Endocrinology*** **158: 1034–1047, 2017)**

Glucocorticoids (GCs) such as cortisol, dexamethasone (DEX), and prednisone are potent anti-inflammatory and immunosuppressive drugs that are widely used for the treatment of chronic conditions, including rheumatoid arthritis, lupus erythematosus, inflammatory bowel disease, asthma, and certain types of leukemia (1). However, long-term use of GCs results in deleterious side effects such as hyperglycemia, hepatosteatosis, and insulin resistance (1). Cushing syndrome and chronic stress can also cause elevated endogenous GC levels that lead to insulin resistance and glucose intolerance (1). The diabetes prevalence in patients taking GCs long term ranges from 20% to 40% (2–4).

GCs exert both their therapeutic and adverse effects through the actions of the GC receptor (GR). The GR belongs to the nuclear hormone receptor family of transcription factors. Ligand activation causes the GR to dissociate from the HSP90/HSP70 complex and translocate to the nucleus. In the nucleus, the GR is recruited to

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Abbreviations: ChIP, chromatin immunoprecipitation; DEX, dexamethasone; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum; *G6pc*, glucose-6-phosphatase; GC, glucocorticoid; *Gilz*, glucocorticoid-induced leucine zipper; GR, glucocorticoid receptor; GRE, glucocorticoid receptor response element; IC_{50}, half maximal inhibitory concentration; IgG, immunoglobulin G; LPS, lipopolysaccharide; LXRα, liver X receptor α; LXRβ, liver X receptor β; mRNA, messenger RNA; *Pepck*, phosphoenolpyruvate carboxykinase; qPCR, quantitative polymerase chain reaction; RNA Pol II, RNA polymerase II; RXR, retinoid X receptor; SCR-1, steroid receptor coactivator 1; SEM, standard error of the mean; TORC2, transcriptional coactivator 2; WT, wild-type.

the regulatory elements of GC-responsive genes to activate or repress transcription by direct binding to a GR response element (GRE) or by indirectly interacting with other transcription factors (1, 5). A key therapeutically desirable effect of GR activation is the potent repression of proinflammatory genes (*i.e., Il1β* and *Il6*) (6). However, GCs also promote hepatic glucose production by increasing transcription of phosphoenolpyruvate carboxykinase (*Pepck*) and glucose-6-phosphatase (*G6pc*), key enzymes involved in gluconeogenesis.

The liver X receptors, LXRα and LXRβ, are sterol sensors that also belong to the nuclear hormone receptor superfamily. LXRα is most highly expressed in the liver, intestine, adipose tissue, kidney, and adrenal glands. In contrast, LXRβ is expressed ubiquitously (7–9). The endogenous ligands of LXRs are oxysterols (10). On activation, LXRs regulate the genes involved in cholesterol efflux (11–13), cholesterol metabolism (14, 15), and fatty acid synthesis (16). The role of LXRs in reverse cholesterol transport (17) has been intensively characterized, and, in line with this function, activation of LXRs was shown to be protective against development of atherosclerosis in mice (18, 19).

Similar to the GR, activation of LXRα or LXRβ suppresses the genes involved in the immune response. Some functions of LXRα and LXRβ do not overlap. For example, the activation of LXRα (but not LXRβ) with synthetic ligands has been shown to improve glycemia in diabetic rodent models by reducing the expression of the gluconeogenic genes, *Pepck* and *G6pc,* in the liver and inducing glucose transporter type 4 (*Glut4*) expression in white adipose tissue (20–26). However, a major deleterious effect of LXRα activation is a potent increase in hepatic lipogenesis, resulting in fatty liver (16, 27). This effect is largely mediated by the LXR target gene and lipogenic transcription factor, *Srebp1c*. In agreement with LXRα having a dominant role in mediating lipogenesis, basal expression of lipogenic genes *Srebp1c*, *Acc*, *Fas*, and *Scd1* are reduced in $Lxr\alpha^{-/-}$ mice (16, 28).

Recently, we demonstrated that whole body $Lxr\beta^{-/-}$ mice are protected against DEX-induced hyperglycemia and hepatosteatosis, but remain sensitive to DEX-mediated inflammatory suppression (29). In mechanistic studies, we showed that LXRβ is essential for full recruitment of the GR to the GRE of *Pepck* after GC administration and that this selective recruitment of GR is target gene dependent (29). To establish whether this finding could be translated into a potential therapeutic strategy to prevent GC-induced diabetes, we performed proof-of-principle experiments combining the administration of a GR agonist with an LXR antagonist. GSK2033 was the first LXRα/β antagonist described in published studies with nanomolar inhibitory potencies in cell-based assays (30). In the present study, we show, using LXR isoform-specific knockout mice, that antagonism of LXRβ (but not LXRα), when combined with GC administration, attenuates gluconeogenic gene activation without affecting the immunosuppressive actions of GR. Taken together, these data suggest a therapeutic approach to dissociate the negative metabolic effects of GC drug therapy from their life-saving anti-inflammatory actions.

Materials and Methods

Reagents

DEX, Dulbecco's modified Eagle medium (DMEM), sodium lactate, penicillin/streptomycin, 0.25% trypsin, and thioglycollate were from Sigma-Aldrich (Oakville, ON, Canada). GSK2033 was synthesized as described previously (30). Fetal bovine serum (FBS), charcoal-treated FBS, liver digestion medium, liver perfusion medium, medium M199, William's E medium, tumor necrosis factor-α (TNF-α), insulin, sodium pyruvate, nonessential amino acids, and Lipofectamine 2000 were purchased from Invitrogen (Burlington, ON, Canada). LXR agonist T0901317 was from Cayman Chemical (Ann Arbor, MI). The antibodies for GR (M-20), RNA polymerase II (C-21), LXRβ (*N*-20), steroid receptor coactivator 1 (SRC-1; M-341), C/EBPβ (C-19), and rabbit immunoglobulin G (IgG) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX). The antibody for TORC2 was from Calbiochem (ST1099; La Jolla, CA). Breeder pairs of LXR-null mice and the following plasmids GAL4-hLXRα, GAL4-hLXRβ, GAL4-hGR, CMX-mLXRα, CMX-mLXRβ, CMX-mGR, UAS-luciferase promoter, and CMX control were provided by Dr. David Mangelsdorf (University of Texas Southwestern Medical Center, Dallas, TX). The rat *Pepck* promoter (−484 to +63, with respect to the transcription start site) was cloned into the PGL3 basic plasmid using rat tail genomic DNA as a template with the forward primer 5′gctcgagagagatctcagagcgtctcg3′ and reverse primer 5′cggggtaccctgaattcccttctcatgacc3′. pNFκB-Luc, consisting of five nuclear factor κB (NF-κB) binding sequences in tandem upstream of a luciferase reporter gene, was a gift from Dr. James Scholey (University of Toronto, ON, Canada) (31).

Mouse primary hepatocytes

Primary hepatocytes were isolated from 7- to 10-week-old wild-type (WT) and *Lxr*-null mice (29) as described in Supplemental Methods. For luciferase reporter assays, the cells were plated in 48-well plates at a density of 8×10^4 cells/well. For gene expression and glucose production analysis, the cells were plated in 6-well plates at a density of 4×10^5 cells/well and 1×10^6 cells/well, respectively. After allowing hepatocytes to adhere overnight, the cells were treated with vehicle, 10 μM GSK2033, 100 nM DEX or DEX plus GSK2033 in M199 without FBS for 16 to 18 hours for gene expression studies. For protein expression and chromatin immunoprecipitation (ChIP) analysis, 4×10^6 cells/treatment were plated in 10-cm plates.

Transient cotransfection reporter assays

Primary hepatocytes grown in 48-well plates were transfected with Lipofectamine 2000 in Opti-MEM (Thermo Fisher

Scientific Life Sciences, Waltham, MA), using the manufacturer's protocol. For the half maximal inhibitory concentration (IC_{50}) experiments with GSK2033, the total amount of plasmid DNA (250 ng/well) included 150 ng UAS-luciferase reporter, 50 ng *Renilla*, 25 ng pCMX-GAL4hLXRα, pCMX-GAL4hLXRβ or pCMX-GAL4hGR, and 25 ng CMX filler plasmid. For *Pepck* promoter activation assays, the total amount of plasmid DNA (250 ng/well) included 125 ng rat Pepck (484 bp) promoter reporter, 50 ng *Renilla*, and the following combinations of nuclear receptors: 25 ng pCMX-mGR and 50 ng CMX; 25 ng pCMX-mGR, 25 ng pCMX-mLXRα, and 25 ng pCMX-mRXRα; and 25 ng pCMX-mGR, 25 ng pCMXm-LXRβ, and 25 ng pCMX-mRXRα. Six hours after transfection, the media were changed to M199 supplemented with 5% charcoal-treated FBS, 1 nM insulin, and 1% penicillin/streptomycin. The next evening, ligands were added in M199 without FBS. Cells were harvested 20 hours later in passive lysis buffer, and the firefly and *Renilla* luciferase activities were measured (Promega, Madison, WI). The firefly luciferase values were normalized to *Renilla* to control for transfection efficiency and are expressed as relative light units.

Glucose production assays

Glucose production experiments were performed as described by Chutkow *et al.* (32). In brief, the hepatocytes were stimulated overnight with indicated ligands in M199 without FBS. Zero-glucose DMEM supplemented with L-glutamine (0.584 g/L), NaHCO3 (3.7 g/L), and HEPES (3.57 g/L), pH 7.3 (glucose-free medium), was prepared. The next day, the cells were washed twice with glucose-free medium and then incubated in glucose-free medium with ligands for 30 minutes to deplete intracellular glycogen. The medium was then replaced with fresh ligand and zero-glucose medium supplemented with 20 mM lactate and 2 mM pyruvate. After 6 hours, the culture medium was removed, lyophilized, and reconstituted to a final volume of 100 μL. The glucose content of the concentrated medium was measured using the glucose hexokinase assay kit (Sigma-Aldrich).

In vivo studies

Lxrα$^{-/-}$ mice [male, 4 to 5 months old, as described by Patel *et al.* (29)] were treated with 5 mg/kg DEX or 5% ethyl alcohol in sesame oil twice daily (subcutaneously) and 40 mg/kg GSK2033 or 5% ethyl alcohol in sesame oil once daily (intraperitoneally) for 5 days alone or in combination. The dose of GSK2033 was chosen from a pilot study in which the LXR agonist T0901317 was injected at 5 mg/kg alone or combined with GSK2033 at 20 or 40 mg/kg, and liver messenger RNA (mRNA) expression of *Abcg5* and *Abcg8* was determined by quantitative polymerase chain reaction (qPCR) (data not shown). To minimize the production of endogenous GCs from environmental stress, the mice were killed in the fed state by decapitation within 1 minute of handling and within 2 hours after the start of the light cycle. Trunk blood was collected in potassium-positive EDTA-containing tubes, and plasma was separated by centrifugation. The tissues were flash frozen in liquid nitrogen for gene expression analysis. The spleen weight was measured. The plasma and tissues were stored at −80°C until assayed. Plasma glucose was measured using a glucose oxidase kit (Wako, Richmond, VA). Plasma insulin was measured by radioimmunoassay (Millipore, Etobicoke, ON, Canada). All animal experiments were approved by the University of Toronto's Faculty of Medicine and Pharmacy Animal Care Committee in accordance with the standards of the Canadian Council on Animal Care.

RNA isolation, complementary DNA synthesis, and real-time QPCR analysis

Total RNA was extracted from cells and tissues using RNA STAT-60 (Friendswood, TX). Following quantification, 2 μg of RNA was treated with DNase I (RNase-free; Invitrogen) and reverse transcribed into complementary DNA with random hexamers using the High Capacity Reverse Transcription System (Applied Biosystems, Burlington, ON, Canada). The primers used are listed in Supplemental Table 1. Real-time quantitative PCR (qPCR) reactions were performed using an ABI 7900 (Applied Biosystems) in 384-well plates containing 12.5 ng complementary DNA, 150 nM of each primer, and 5 μL 2X SYBR Green PCR Master Mix (Applied Biosystems) in a 10-μL total volume. Relative mRNA levels were calculated using the comparative Ct method (33).

Protein extraction and Western blotting

Nuclear and cytoplasmic fractions were prepared from flash-frozen liver pieces as described in Supplemental Methods. Nuclear extracts (50 μg) or whole cell lysates (50 μg) were electrophoresed on 4% to 20% gradient gels (Bio-Rad Laboratories, Berkeley, CA) and transferred to nitrocellulose using standard techniques. The blot was blocked for 1 hour in 5% nonfat milk and incubated overnight at 4°C with primary polyclonal GR (M-20) antibody (1:2000; Santa Cruz Biotechnology), GAPDH (1:15,000; Abcam, Cambridge, MA), Pepck (1:1000; Abcam), actin (1:5000; Abcam) in 1% nonfat milk. The membrane was washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 and incubated with secondary horseradish peroxidase-conjugated anti-rabbit IgG (1:5000) or anti-goat IgG (1:5000) for 1 hour. The membrane was washed three times with PBS containing 0.05% Tween-20, and the signal was observed using ECL prime (GE Healthcare, Mississauga, ON, Canada). The blots were quantified using ImageJ software (National Institutes of Health, Bethesda, MD). Three individual sets of experiments and Western blots were performed.

ChIP analysis

Lxrα$^{-/-}$ livers were perfused *in situ* for 30 minutes via the portal vein with vehicle, 10 μM GSK2033, 100 nM DEX or DEX plus GSK2033 dissolved in perfusion buffer. ChIP was performed as described in Supplemental Methods. Quantitation was performed by qPCR using the standard curve method with serial dilutions of a 10% input as standard. The results were normalized to 3% total chromatin input.

Mouse primary macrophage and RAW264.7 studies

WT and *Lxr*-null mice were injected intraperitoneally with 1 mL of 3% thioglycollate 4 days before macrophage isolation. After sacrificing the mouse, macrophages were collected by peritoneal lavage using 10 mL of cold PBS. Isolated cells were pelleted at 1700*g* and washed in PBS supplemented with 3% FBS three times. The cells were resuspended in high-glucose DMEM supplemented with 10% FBS and 100 U/mL penicillin/streptomycin, counted,

assessed for viability, and plated to a density of 2×10^6 cells/well in 6-well plates. After allowing the macrophages to adhere overnight, pretreatment with 10 ng/mL LPS was initiated. Four hours later, the cells were cotreated with the indicated concentrations of drug for 16 to 18 hours. The next morning, the cells were harvested for RNA.

RAW264.7 cells were grown in high-glucose DMEM supplemented with 10% FBS and 100 U/mL penicillin/streptomycin. Ligand treatment was performed as described previously.

Statistical analysis

Data are presented as the average ± standard error of the mean (SEM), unless otherwise indicated. GraphPad Prism was used for analysis of variance followed by Newman-Keuls test. To compare two groups, the two-tailed Student *t* test was used. $P < 0.05$ was considered statistically significant.

Results

GSK2033 is a dual LXR antagonist

GSK2033 was reported by Zuercher *et al.* (30) in 2010 to be a potent LXR antagonist (IC_{50} of 100 nM for GAL4-LXRα and 40 nM for GAL4-LXRβ) in cell-based assays. We confirmed this level of activity in HEK293 cells expressing full-length receptors (IC_{50} of 310 nM for LXRα and 83 nM for LXRβ) (Supplemental Fig. 1). However, the utility of the compound for *in vivo* studies was questioned, because GSK2033 was rapidly metabolized in human liver microsomes (30). Therefore, to determine an optimal concentration for dosing in our studies using mice, we assessed GSK2033's potency and selectivity in mouse primary hepatocytes (Fig. 1). In cotransfection reporter studies, GSK2033 potently antagonized the T0901317-induced activation of GAL4-LXRα/β without affecting the DEX-induced activity of GAL4-GR. GSK2033 showed preferential selectivity for LXRβ, with an IC_{50} of 167 nM, compared with LXRα, with an IC_{50} of 1.7 μM. This difference in potencies was not anticipated to be sufficient to selectively inhibit LXRβ in cells; thus, our studies were performed using LXR isoform-specific knockout mice.

LXRβ antagonism impairs GC-induced gluconeogenesis in mouse primary hepatocytes

A major undesirable side effect of GC treatment is increased glucose production by the liver. To investigate whether antagonism of LXRβ, along with DEX treatment, alters GR-mediated *Pepck* expression and glucose output *in vitro*, we conducted gene expression and glucose production studies in mouse primary hepatocytes isolated from WT, *Lxrα*$^{-/-}$ and *Lxrβ*$^{-/-}$ mice. As expected, DEX treatment significantly increased the expression of *Pepck*, a key gluconeogenic gene, in WT and *Lxrα*$^{-/-}$ primary hepatocytes [Fig. 2(a)]. The induction of *Pepck* by DEX was significantly inhibited by cotreatment with GSK2033 in both WT (from 8.5-fold to 1.9-fold) and *Lxrα*$^{-/-}$ (from 15.7-fold to 3.2-fold) hepatocytes. In agreement with our previous studies, DEX treatment did not increase *Pepck* expression in *Lxrβ*$^{-/-}$ primary hepatocytes, and this remained unchanged with cotreatment of GSK2033 [Fig. 2(a)]. In contrast, the expression of another GR target gene, *Tat*, was significantly induced by DEX in WT (20.8-fold), *Lxrα*$^{-/-}$ (12.7-fold), and *Lxrβ*$^{-/-}$ (9.7-fold) hepatocytes and unchanged with GSK2033 cotreatment [Fig. 2(b)]. As expected, the LXR target gene *Srebp1c* was induced by T0901317 in hepatocytes from WT (37.7-fold), *Lxrα*$^{-/-}$ (3.7-fold) and *Lxrβ*$^{-/-}$ (42-fold) mice, and this induction was significantly downregulated by GSK2033 cotreatment, independent of genotype [Fig. 2(c)]. To determine whether GSK2033 was indirectly contributing to the suppression of DEX-mediated *Pepck* expression through the regulation of another protein, we performed similar experiments in the presence of the protein synthesis inhibitor cycloheximide. Under these conditions, GSK2033 maintained its ability to

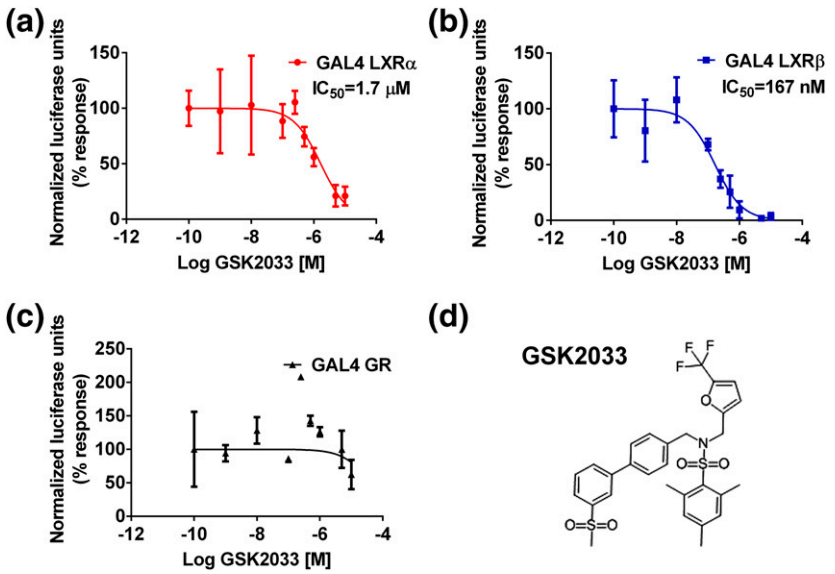

Figure 1. GSK2033 is a potent dual LXR antagonist in mouse primary hepatocytes. Mouse primary hepatocytes were cotransfected with GAL4-LXRα, GAL4-LXRβ, or GAL4-GR and a UAS-luciferase reporter plasmid. A dual LXRα/β ligand, T0901317, was dosed at the 80% effective concentration for each receptor (1 μM for GAL4-LXRα and 250 nM for GAL4-LXRβ). The IC_{50} of GSK2033 was determined for LXRα (a) and LXRβ (b) in mouse primary hepatocytes. (c) The activity of GSK2033 was assessed against GAL4-GR activated with 100 nM DEX. (d) Chemical structure of GSK2033. Data presented as average ± SEM (n = 3). Similar results were obtained with three different preparations of hepatocytes. The IC_{50} values were determined by curve fitting using the log(inhibitor) vs normalized response function (Prism, GraphPad).

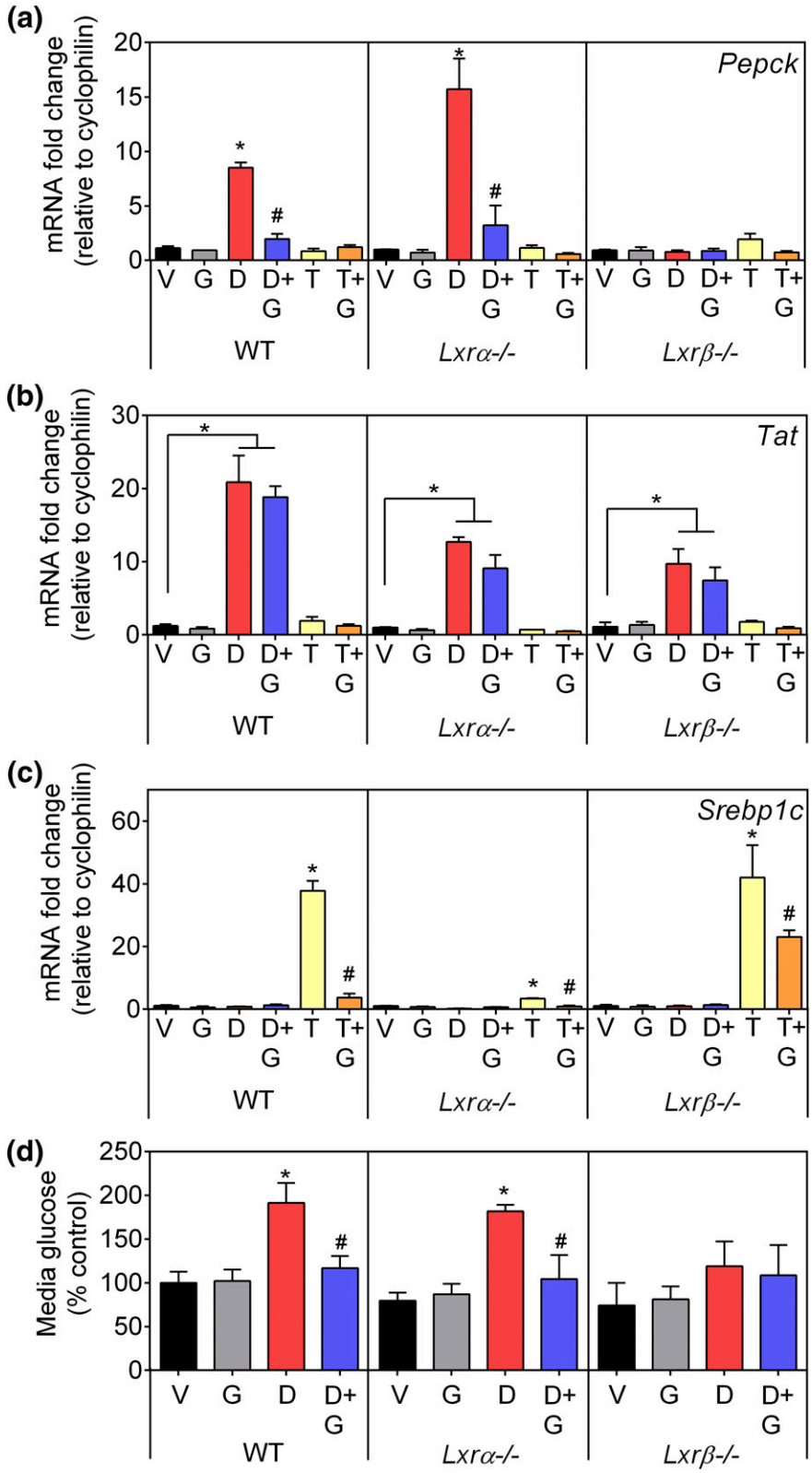

Figure 2. LXRβ antagonism prevents DEX-induced *Pepck* expression and glucose production in mouse primary hepatocytes. Expression of the GR target genes (a) *Pepck* and (b) *Tat* and the LXR target gene (c) *Srebp1c* in WT, *Lxrα*$^{-/-}$, and *Lxrβ*$^{-/-}$ mouse primary hepatocytes after 18 hours of treatment with vehicle (V), 10 μM GSK2033 (G), 250 nM T0901317 (T; LXR agonist), 100 nM DEX (D) alone or in combination. (d) Glucose output over 6 hours from WT, *Lxrα*$^{-/-}$, and *Lxrβ*$^{-/-}$ hepatocytes treated with 500 nM DEX with or without 10 μM GSK2033. (a–c) Data presented as average ± SD, n = 3, repeated four to five times. (d) Data presented as average ± SEM, n = 3 to 5 independent experiments. *$P < 0.05$ V vs D or T and $^{\#}P < 0.05$ D vs D plus G or T vs T plus G of the same genotype by one-way analysis of variance and Newman-Keuls test.

Table 1. Physiologic Parameters of $Lxr\alpha^{-/-}$ Mice After DEX With or Without GSK2033 Treatment

Variable	Vehicle	DEX	DEX Plus GSK2033
Body weight change, %	−1 ± 2	−3 ± 1	2 ± 2
Plasma glucose, mg/dL	107 ± 6	140 ± 20	110 ± 10
Plasma triglycerides, mg/dL	71 ± 5	82 ± 4	79 ± 8
Plasma insulin, ng/mL	1.8 ± 0.2	40 ± 7[a]	29 ± 6[a]
Normalized liver weight, g/g	0.038 ± 0.002	0.049 ± 0.002[a]	0.046 ± 0.002[a]
Liver triglyceride, mg/g tissue	22 ± 9	32 ± 6[a]	15 ± 3[b]

Data presented as the average ± SEM; n = 5 to 7.

$Lxr\alpha^{-/-}$ mice were treated with vehicle or DEX 5 mg/kg twice daily with or without GSK2033 40 mg/kg intraperitoneally once daily.

[a] $P < 0.05$, vehicle vs DEX.

[b] $P < 0.05$ DEX vs DEX plus GSK2033, by one-way analysis of variance and Newman-Keuls test.

diminish DEX-induced *Pepck* expression in $Lxr\alpha^{-/-}$ primary hepatocytes, suggesting that *de novo* protein synthesis is not required to exert this inhibitory effect [Supplemental Fig. 2(a)]. In addition, the effects of SK2033 on gluconeogenic gene inhibition was not limited to DEX; GSK2033 cotreatment with other GCs such as cortisol, prednisone, and triamcinolone acetonide also repressed the expression of *Pepck* in $Lxr\alpha^{-/-}$ primary hepatocytes [Supplemental Fig. 2(b) and 2(c)]. In summary, these results indicate that antagonism of LXRβ is able to attenuate GC-mediated upregulation of the gluconeogenic program independent of *de novo* protein synthesis in mouse primary hepatocytes.

Glucose production studies of hepatocytes confirmed the functional importance of the changes we observed in gluconeogenic enzymes. As expected, only WT and $Lxr\alpha^{-/-}$ primary hepatocytes were able to secrete substantial glucose in the medium with DEX treatment, and GSK2033 significantly attenuated DEX-stimulated glucose production in WT and $Lxr\alpha^{-/-}$ primary hepatocytes [Fig. 2(d)]. These results demonstrate that antagonism of LXRβ is sufficient to inhibit GC-induced *de novo* glucose production in hepatocytes.

LXRβ antagonism represses GC-mediated induction of gluconeogenesis *in vivo*

To assess the translatability of our findings from mouse primary hepatocytes, we performed an *in vivo* study in $Lxr\alpha^{-/-}$ mice in which GSK2033 was dosed by intraperitoneal injection. Reasoning that GSK2033 is rapidly metabolized by the liver, we hoped to capture the early liver-mediated effects of LXRβ antagonism using this dosing method. $Lxr\alpha^{-/-}$ mice were injected subcutaneously with 5 mg/kg DEX (twice daily) and intraperitoneally with 40 mg/kg GSK2033 once daily for 5 days and killed at lights on (representing the nadir of endogenous GC levels in mice). Body weight and plasma triglyceride levels were unchanged in all groups, although the plasma glucose levels tended to increase with DEX treatment (Table 1). In contrast, DEX significantly increased circulating insulin levels and liver weights, which remained unaffected by GSK2033 cotreatment. DEX increased hepatic triglyceride accumulation 1.5-fold more than vehicle ($P < 0.05$), and coadministration with GSK2033 completely reversed this GC-induced hepatic steatosis (Table 1), in line with the known basal role for LXRs in hepatic lipogenesis (16, 28).

Liver gene expression analyses found that DEX significantly increased the expression of gluconeogenic genes (*Pepck*, *G6pc*, *Pgc1α*, and *Foxo1*), and this induction was significantly diminished when mice were cotreated with GSK2033. This effect was, in part, gene selective because *Per1* and *Igfbp1,* two nongluconeogenic GR target genes, were increased with DEX but unaffected by GSK2033 cotreatment [Fig. 3(a)]. The expression of the nuclear receptors *Gr* and *Lxrβ* remained unchanged by the treatments, indicating that *Gr* and *Lxrβ* do not regulate each other's expression [Fig. 3(a)]. In agreement with the mRNA expression pattern for *Pepck*, hepatic PEPCK protein expression was also decreased when GSK2033 was coadministered with DEX compared with DEX treatment alone [Fig. 3(b) and 3(c)]. We also assessed the effect of GSK2033 in WT mice treated with DEX for 10 days. Similar to our results in $Lxr\alpha^{-/-}$ mice, GSK2033 suppressed the DEX-mediated induction of *Pepck*. In contrast, we saw no substantial changes with DEX or GSK2033 on *G6pc* or *Foxo1* expression levels [Supplemental Fig. 3(a)].

Under basal conditions, GR is found in the cytoplasm complexed to heat shock proteins. With the addition of DEX, binding of the ligand to GR causes a conformational change, resulting in the nuclear translocation of GR (34). Therefore, altering the nuclear translocation of GR is one mechanism by which the GC/GR liver response can be modulated. In our *in vivo* study, we measured GR protein from nuclear and cytoplasmic fractions and, as expected, found that DEX significantly increased nuclear translocation of GR. We were surprised to find that

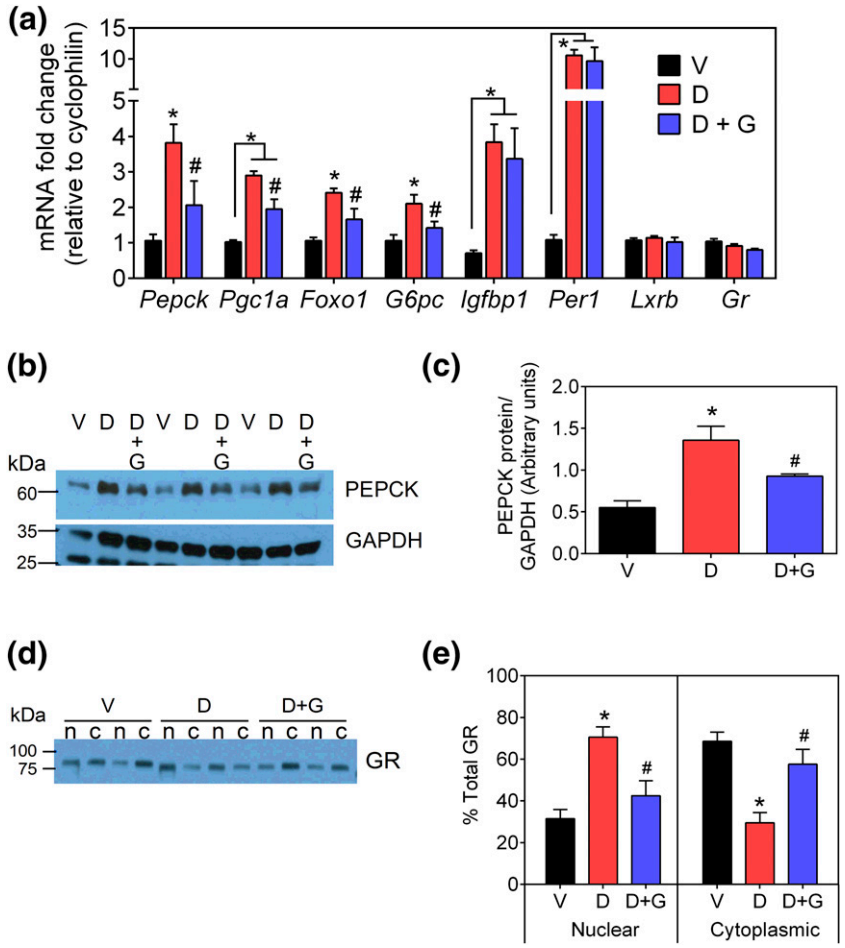

Figure 3. *Lxrα*$^{-/-}$ mice treated with DEX plus GSK2033 have reduced gluconeogenic gene expression and decreased GR nuclear translocation compared with mice treated with DEX alone. *Lxrα*$^{-/-}$ mice were treated with vehicle (V), 5 mg/kg twice daily DEX (D) with or without 40 mg/kg GSK2033 (G) for 5 days. (a) Liver gene expression of *Pepck*, *Pgc1a*, *Foxo1*, *G6pc*, *Igfbp1*, *Per1*, *Lxrβ*, and *Gr* and measured by qPCR (n = 5 to 7). (b) Liver PEPCK protein levels and (c) quantitation of PEPCK normalized to GAPDH levels (n = 3 to 4). (d) Liver GR protein expression in nuclear (n) and cytoplasmic (c) fractions and (e) quantitation of GR relative to total GR (n plus c) from each sample (n = 3 to 4). (a) Data presented as average ± SEM; $^{*}P < 0.05$ D vs V and $^{\#}P < 0.05$ D vs D + G by one-way analysis of variance and Newman-Keuls test.

nuclear GR translocation was significantly diminished in mice treated with DEX plus GSK2033 compared with DEX alone in *Lxrα*$^{-/-}$ [Fig. 3(d) and 3(e)] and WT [Supplemental Fig. 3(b)] mice. Furthermore, GSK2033 treatment alone had no effect on GR translocation [Supplemental Fig. 3(c)]. The change in localization of GR with DEX plus GSK2033 was unexpected because our previous studies of GR translocation in response to DEX in *Lxrβ*$^{-/-}$ mice had shown no differences compared with WT mice (29). In addition, when we explored the effect of DEX vs DEX plus GSK2033 on GR translocation by overexpressing Cherry-LXRβ and Venus-GR in primary hepatocytes, we saw no preferential nuclear exclusion of GR with DEX plus GSK2033 treatment (Supplemental Fig. 4). Thus, decreased nuclear translocation of GR could be one mechanism leading to downregulation of gluconeogenesis only in our *in vivo* study. In conclusion, these *in vivo* data complement our *in vitro* results indicating that LXRβ antagonism selectively attenuates the GR target genes involved in gluconeogenesis.

LXRβ antagonism interferes with GR-mediated activation in a promoter-specific manner

To assess whether the interplay between LXRβ and GR was occurring at the level of the promoter, we conducted cotransfection studies in WT primary hepatocytes using the rat *Pepck* (484 bp) promoter reporter. As expected, DEX treatment increased the activity of the *Pepck* promoter reporter in cells transfected with GR alone or combined with LXRα/β and the retinoid X receptor (RXRα). More importantly, this DEX-mediated activation was significantly diminished by cotreatment with GSK2033 only in hepatocytes cotransfected with the combination of GR, LXRβ, and RXRα, but not with the combination of GR, LXRα, and RXRα or GR alone [Fig. 4(a)]. Antagonism of LXRα increased the activity of the *Pepck* promoter reporter, in agreement with published reports detailing the negative regulation of *Pepck* by LXRα (20, 23). Together, these results confirm the isoform-specific roles for LXRα vs LXRβ in the regulation of the *Pepck* promoter and suggest that LXRβ antagonism disrupts a transcriptional complex required for GR-mediated *Pepck* activation.

To assess the consequence of LXR antagonism on GR-mediated transrepression, we conducted similar cotransfection studies in HEK cells using a luciferase reporter expressing 5 copies of the NF-κB response element. TNF-α was used to activate NF-κB signaling, and, as expected, DEX repressed the NF-κB responsive reporter activity equally in cells transfected with GR alone or combined with either of the LXR isoforms and RXRα. Importantly, this DEX-mediated repression was not adversely affected by cotreatment with GSK2033 [Fig. 4(b)]. GSK2033 repressed TNF-α–induced NF-κB reporter expression even in the absence of DEX. Taken together, these results imply that LXRβ antagonism affects GR-mediated transcriptional activity in a target gene promoter-specific manner.

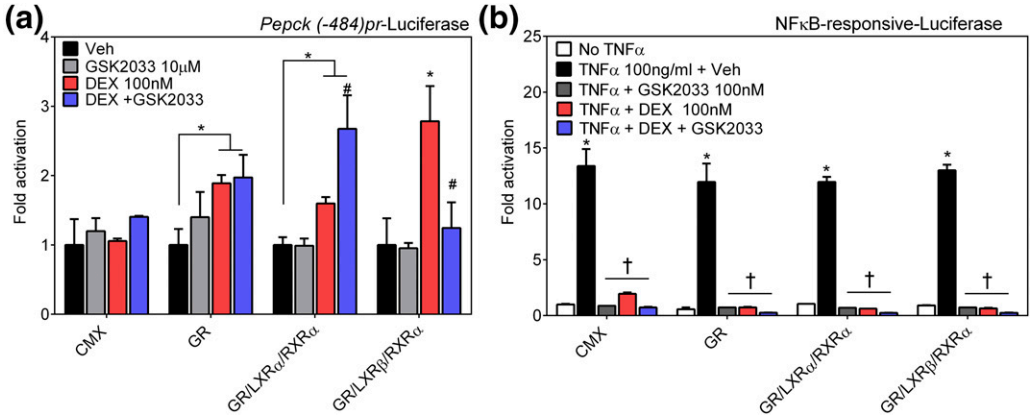

Figure 4. LXRβ antagonism attenuates GR-mediated transcriptional activity at the Pepck promoter. (a) Mouse primary hepatocytes were cotransfected with *Pepck* promoter luciferase along with the indicated combinations of receptors and *Renilla* control plasmid. After 18 hours of treatment with 100 nM DEX with or without 10 μM GSK2033, *Pepck* (−484 bp) promoter reporter activity was measured using the dual luciferase reporter kit. (b) HEK293 cells were cotransfected with a NF-κB responsive luciferase reporter with the indicated combinations of receptors and β-galactosidase control plasmid. After 18 hours of treatment with vehicle (Veh), 100 ng/mL TNF-α with or without 100 nM DEX with or without 100 nM GSK2033, NF-κB activity was measured. Data presented as average ± SD, n = 3, repeated 2 times; $^{*}P < 0.05$ compared with Veh; $^{\#}P < 0.05$, DEX vs DEX plus GSK; $\dagger P < 0.05$ vs TNF-α by one-way analysis of variance and Newman-Keuls test.

GSK2033 impairs recruitment of GR, C/EBPβ, and MED1 to the Pepck promoter

To better define how antagonism of LXRβ was selectively modulating the transcription factor recruitment near the proximal *Pepck* promoter, we conducted ChIP studies in *Lxr*$\alpha^{-/-}$ livers perfused *in situ* via the portal vein with vehicle or DEX with or without GSK2033 for 30 minutes. This method of perfusion was chosen, because it allowed us to obtain samples from an *in vivo* context without the complicating influence of counter-regulatory hormones present in the circulation. Given the direct interplay of LXRβ and GR on the activity of the −484-bp *Pepck* promoter [Fig. 4(a)], we first determined whether LXRβ was recruited to this region. With ChIP, we were unable to observe any substantial recruitment of LXRβ beyond background levels at the *Pepck* proximal promoter. In contrast, strong recruitment of LXRβ was found at the LXR response element of the *Srebp-1c* promoter [Fig. 5(a)]. Although it is formally possible that LXRβ could be at the *Pepck* promoter with its epitope masked as a result of a protein complex, these data suggest that LXRβ is not significantly recruited to the proximal *Pepck* promoter in response to DEX or GSK2033.

The *Pepck* promoter has two low-affinity GR binding sites (−387 to −374 and −367 to −353 bp from the transcription start site) (35); therefore, the presence and proper assembly of several accessory proteins [including hepatocyte nuclear factor-α, cyclic adenosine monophosphate response element-binding protein, C/EBPβ, transcriptional coactivator 2 (TORC2), FOXO1, peroxisome proliferator-activated receptor γ-coactivator 1-α, MED1, and SRC-1] is requisite for potent induction of *Pepck* after GC treatment (36–38). Using ChIP, we observed a robust recruitment of GR to both *Pepck* and *Tat* GREs in response to DEX [Fig. 5(b)]. Strikingly, GR occupancy of the *Pepck* GRE, but not the *Tat* GRE, was significantly diminished when GSK2033 was coadministered with DEX [Fig. 5(b)]. Moreover, DEX increased the recruitment of MED1, the GR coactivator and mediator complex subunit, and RNA polymerase II (RNA Pol II) to the *Pepck* promoter. The recruitment of MED1 and RNA Pol II was attenuated when livers were perfused with GSK2033 plus DEX compared with DEX alone [Fig. 5(c) and 5(d)]. We also observed substantial recruitment of SRC-1, the cyclic adenosine monophosphate response element-binding protein regulated TORC2, and C/EBPβ to the *Pepck* GRE after DEX perfusion; however, of these accessory proteins, only C/EBPβ occupancy was attenuated by coperfusion of GSK2033 with DEX [Fig. 5(e)].

Taken together, the findings from ChIP and *Pepck* promoter reporter studies support the idea that LXRβ antagonism destabilizes the GR transcriptional complex at the *Pepck* promoter resulting in reduced GC-induced *Pepck* expression.

GC-mediated immune suppression is not affected by GSK2033

The immunosuppressive and anti-inflammatory effects of GCs are achieved by two mechanisms: the direct or indirect repression of proinflammatory genes and the transactivation of anti-inflammatory genes. To determine whether GSK2033 would affect GR-mediated inflammatory responses, we conducted gene expression studies using primary thioglycollate-elicited peritoneal macrophages isolated from WT, *Lxr*$\alpha^{-/-}$, and *Lxr*$\beta^{-/-}$ mice. As expected, DEX potently decreased LPS-induced

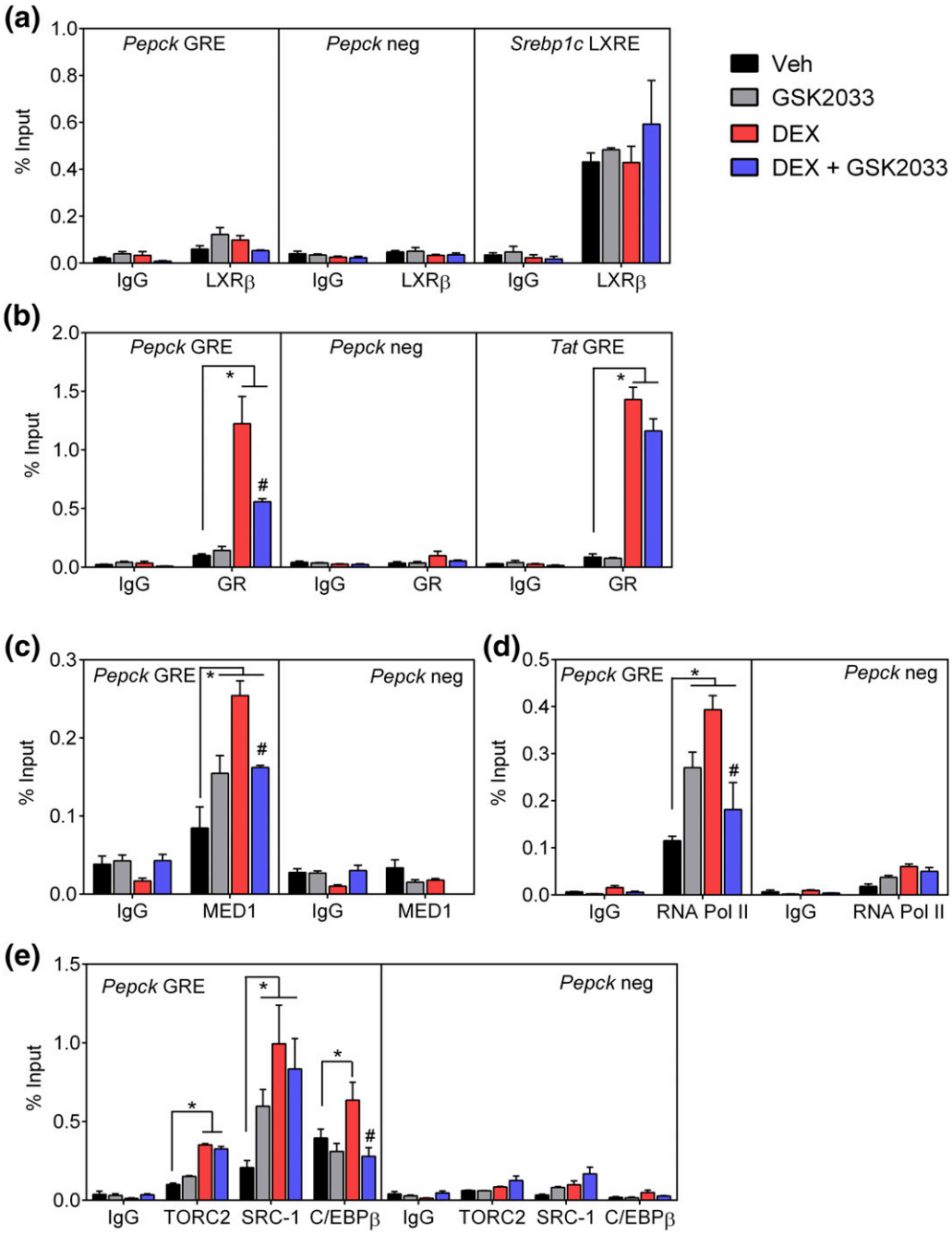

Figure 5. GSK2033 decreases recruitment of GR, MED1, and C/EBP1 to the *Pepck* promoter in *Lxrα*$^{-/-}$ livers. ChIP analyses of *Lxrα*$^{-/-}$ livers after 30 minutes of perfusion of 100 nM DEX with or without 10 μM GSK2033 via the portal vein. The results are expressed relative to the percentage of input. (a) LXRβ recruitment to the *Pepck* GRE, *Pepck* promoter-negative control region, and *Srebp1c* LXRE. (b) GR recruitment to the *Pepck* GRE, *Pepck* promoter-negative control region, and *Tat* GRE. (c) MED1, (d) RNA Pol II, (e) TORC2, SRC-1, and C/EBPβ recruitment to the *Pepck* GRE, *Pepck* promoter-negative control region. Data are from a representative experiment (average ± SD, qPCR error) repeated in independent liver samples at least three times. *$P < 0.05$ vehicle (Veh) vs DEX or GSK2033; $^{\#}P < 0.05$ DEX vs DEX plus GSK2033 by one-way analysis of variance and Newman-Keuls test.

expression of the proinflammatory genes, *Il1β* and *Il6*, regardless of genotype. Importantly, DEX-mediated *Il1β* and *Il6* repression was unaltered by GSK2033 cotreatment [Fig. 6(a)]. Because of the very strong suppression of proinflammatory gene expression by 100 nM DEX in primary macrophages, we performed additional dose–response experiments with lower concentrations of DEX in primary macrophages and RAW264.7 cells (0.1 to 100 nM) and found that, even at the lowest dose of DEX tested (0.1 nM), GSK2033 did not interfere with the ability of DEX to inhibit *Il1β* expression (Supplemental Fig. 5). Moreover, the induction of the anti-inflammatory gene GC-induced leucine zipper (*Gilz*) by DEX was unaffected by GSK2033 cotreatment [Fig. 6(a)]. Thus,

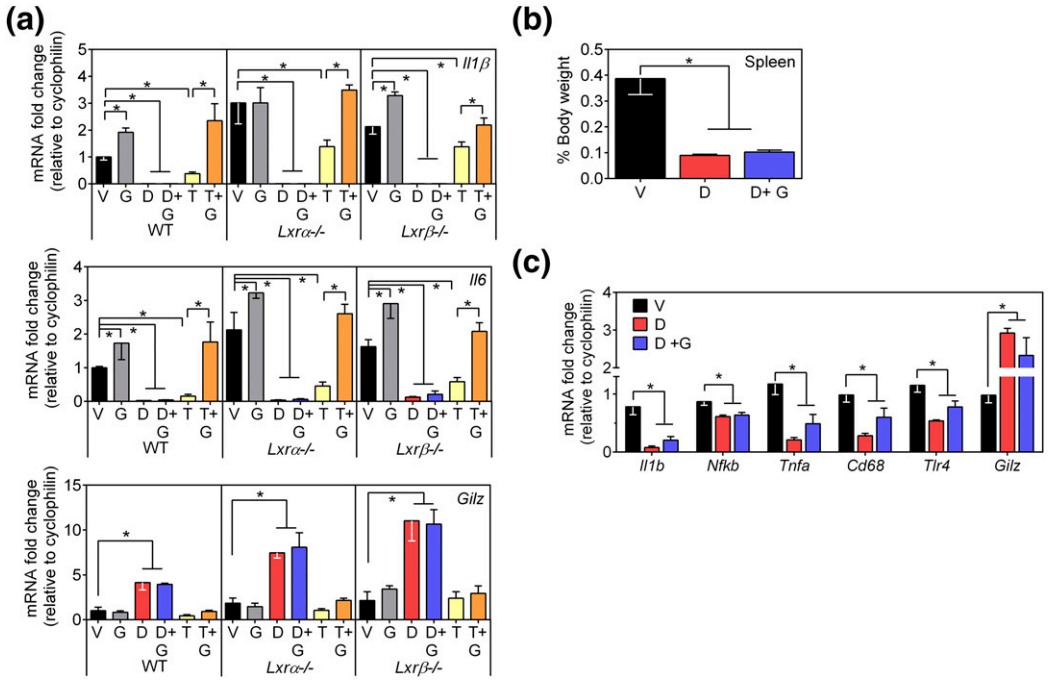

Figure 6. GR-mediated immune suppression is not affected by GSK2033. (a) LPS activated WT, *Lxrα*$^{-/-}$ and *Lxrβ*$^{-/-}$ mouse primary macrophages were treated with vehicle (V), 10 μM GSK2033 (G), 250 nM T09 (LXR agonist), 100 nM DEX (D) alone or combined for 18 hours before qPCR analysis of genes important for immune suppression (*Il1β*, *Il6*, and *Gilz*). (b) Spleen weight and (c) hepatic expression of immunomodulatory genes *Il1β, Nfκβ, Tnfα, Cd68, Tlr4*, and *Gilz* in *Lxrα*$^{-/-}$ treated with vehicle (V), DEX (D) 5 mg/kg twice daily with or without GSK2033 (G) 40 mg/kg (intraperitoneally daily) for 5 days. (a) Data presented as average ± SD, n = 3, repeated three independent times. (b, c) Data presented as average ± SEM, n = 5 to 7; $*P < 0.05$ by one-way analysis of variance and Newman-Keuls test.

GC-mediated immune suppression is unaffected by LXR antagonism in mouse primary macrophages. In agreement with the findings reported by several groups (39–42), treatment with the LXR agonist T0901317 decreased the expression of *Il1β* and *Il6*, and this suppression was antagonized by cotreatment with GSK2033 [Fig. 6(a)]. These data strongly support the idea that although GSK2033 is targeting LXR*β* in primary macrophages, it did not antagonize the anti-inflammatory actions of GCs in this system.

To assess the *in vivo* consequence of LXR*β* antagonism with respect to GC-mediated effects on the immune system, we measured the spleen weight and liver expression of immune-responsive genes in *Lxrα*$^{-/-}$ mice administered DEX or DEX plus GSK2033 for 5 days. Spleen atrophy is a well-known marker of immune suppression after GC treatment (43, 44). The spleen weights in mice treated with DEX or DEX plus GSK2033 were significantly decreased compared with vehicle-treated mice, indicating immune suppression was maintained in mice treated with the LXR*β* antagonist [Fig. 6(b)]. Additionally, proinflammatory gene expression in the liver (*Il1β*, *Nfkβ*, *Tnfα*, *Cd68*, and *Tlr4*) was significantly suppressed by DEX, and this suppression was not affected by cotreatment with GSK2033 [Fig. 6(c)]. The induction of the anti-inflammatory gene *Gilz*, in response to DEX, was also unchanged in the presence of GSK2033 [Fig. 6(c)]. Taken together, these data show that LXR*β* antagonism spares the desired immunosuppressive actions of GC administration.

Discussion

Each year, 10 million new prescriptions are written for oral GCs in the United States, and the odds ratio for new-onset diabetes in GC-treated patients ranges from 1.5 to 2.5 (45). The pharmaceutical industry has long been interested in developing GC therapeutic agents devoid of metabolic side effects. However, the traditional view that the immunosuppressive actions of GCs are mediated by transrepression of inflammatory genes (*Il1β*, *Il6*) and that the metabolic side effects are mediated by transactivation of metabolic genes (*Pepck*, *G6pc*) was too simplistic, because the potent immunosuppressive actions of GCs also requires the transactivation of anti-inflammatory genes (*Gilz*, *Dusp14*) (46). An alternate strategy to achieve dissociated GC effects could be to combine the administration of a GC drug with another therapeutic agent (not targeting GR) that can mitigate the gluconeogenic side effects of GCs without altering their anti-inflammatory activity. In our study, we found that LXR*β* antagonism achieves this effect in mice treated with DEX. The role for LXR*β* in this context is surprising because LXR*β* has not traditionally been considered to play a

substantial role in the liver (relative to LXRα) and because LXRα and LXRβ appear to play opposing roles in the regulation of hepatic gluconeogenesis, but redundant roles in the regulation of inflammatory gene expression.

Previous studies by several groups have shown that agonism of LXRα is effective at attenuating gluconeogenic gene expression induced by diabetes (20, 22) or GC treatment (24). In agreement with Nader *et al.* (24), we found that cotreatment of DEX with T0901317 (an LXR agonist) was able to attenuate *Pepck* expression in WT hepatocytes but not in *Lxrα*$^{-/-}$ hepatocytes [Supplemental Fig. 6(a)]. Moreover, glucose production in WT hepatocytes was significantly decreased after T0901317 cotreatment with DEX [Supplemental Fig. 6(b)], demonstrating an inhibitory role for LXRα. Transient cotransfection studies showed that antagonism of LXRβ caused decreased activation of the *Pepck* promoter. In contrast, antagonism of LXRα caused enhanced activation of the *Pepck* promoter. These results suggest both LXRα and LXRβ are functionally important in mouse hepatocytes for the regulation of GC-induced gluconeogenesis. Because of this dichotomous relationship, we were intrigued to discover that when WT hepatocytes were cotreated with DEX and GSK2033 at a dose that inhibited both LXRα and LXRβ, *Pepck* expression and glucose production were suppressed. Likewise, when WT mice were treated with DEX plus GSK2033, we found that *Pepck* expression was diminished relative to DEX treatment alone. Together, these data support opposing roles for LXRα vs LXRβ in gluconeogenesis. Furthermore, it suggests that when DEX is given in the presence of a pan-LXR agonist (T0901317), the actions of LXRα predominate and, in the presence of a pan-LXR antagonist, the function of LXRβ predominates.

Our perfused liver ChIP studies revealed that coadministration of GSK2033 with DEX decreased GR, MED1, C/EBPβ, and RNA Pol II recruitment to the *Pepck* proximal promoter without changing the recruitment of other coregulators (SRC-1 and TORC2) to the same site. In addition, within the same samples, we saw no change in the recruitment of GR to the *Tat* promoter in the presence of GSK2033. We also did not observe substantial recruitment of LXRβ to the *Pepck* proximal promoter. We investigated whether LXRβ and GR form a protein complex, but we did not detect a physical association between GR and LXRβ using unbiased proteomic approaches (*i.e.,* HEK293 cells stably overexpressing either BirA-LXRβ or BirA-GR constructs or co-immunoprecipitation analyses of endogenous liver LXRβ and GR from *Lxrα*$^{-/-}$ mice administered DEX with or without GSK2033). However, we did find it possible to detect a protein–protein interaction between overexpressed tagged-LXRα/tagged-LXRβ and tagged GR, indicating that an interaction is possible under these artificial conditions but this interaction is not LXR isoform selective (Supplemental Fig. 7).

How then, does LXRβ confer GR gene responsiveness in a target gene selective manner? Several factors are likely contributing to the selective regulation of GR target genes in response to LXRβ antagonism. These include (1) the chromatin context and the accessibility of GR to DNA, (2) the presence of coregulatory binding factors, and (3) the strength of the GR binding site. It is well established that many GR-regulated genes are made of composite elements requiring transcription factors, apart from GR, to confer a full hormonal response (47, 48). The *Pepck* gene is an example of one such promoter that has weak GREs and requires the contribution of multiple transcription factors within the GC response unit to elicit a maximal response (37, 49, 50). The combination of distinct cofactors important at different promoters, the sequence of the GRE, and the allosteric effect of the ligand on DNA binding are all factors that will influence the strength of the GR–DNA binding interaction (5, 34, 51).

A recent mouse liver ChIP-sequence study showed that 62% of GR-binding sites are occupied by C/EBPβ. The findings from that study suggested a priming role for C/EBPβ in genome-wide GR recruitment, because disruption of C/EBPβ binding to the chromatin resulted in attenuation of GR recruitment to GR-binding sites (51). Additionally, MED1 is an important regulator of GR transcriptional activity, because hepatic *Pepck* expression is not induced in liver-specific *Med1*$^{-/-}$ mice after DEX treatment (38). These studies imply an important role for C/EBPβ and MED1 in GC-mediated induction of *Pepck*. Our results suggest that GC-induced MED1, C/EBPβ, and GR interactions at the proximal *Pepck* promoter are disrupted by the LXRβ antagonist GSK2033, leading to decreased promoter engagement and RNA Pol II recruitment and attenuation of *Pepck* expression. Our data support the idea that LXRβ helps stabilize the full complement of GR accessory factors within this GC responsive transcriptional complex.

A key finding from our study was that combination treatment of DEX plus GSK2033 did not alter spleen weight *in vivo* relative to DEX alone nor modify the gene expression profiles of immune-responsive GR targets in mouse primary macrophages. These data indicate that GR-mediated immune suppression (a desired effect of GC therapy) is not affected by LXR antagonism. This is consistent with our reported finding in *Lxrα*$^{-/-}$ and *Lxrβ*$^{-/-}$ mice, showing that LXRs are dispensable for GC-mediated immune suppression (29).

The results from our group, and others, suggest that both agonism of LXRα and antagonism of LXRβ are effective at attenuating the GC-induced gluconeogenic

program. A major pitfall associated with use of LXRα activation to control hyperglycemia is the induction of hepatic *de novo* lipogenesis via activation of *Srebp1c*, *Fas*, and *Scd1*. LXRα is highly expressed in the liver (7) and is the dominant LXR isoform influencing hepatic lipogenesis [Fig. 2(c)] (16, 27, 52, 53). Thus, long-term use of LXRα agonists to attenuate GC-induced gluconeogenesis would aggravate hepatosteatosis, which itself is another detrimental side effect of GC administration. In contrast, the use of LXRβ antagonists to attenuate the glycemic effects of GCs would not cause the same lipogenic effects associated with LXRα activation. We observed that GC-mediated hepatosteatosis was lowered by LXRβ antagonism (Table 1). However, a potential caveat to the use of LXRβ antagonists is the atheroprotective role that both LXR isoforms share. LXRα and LXRβ are equally important for reverse cholesterol transport (through activation of ABCA1) in the peripheral organs (17, 54). One strategy to overcome this potential liability is to develop an LXRβ-specific antagonist that would be primarily taken up by the liver (high first-pass metabolism), where GC-induced gluconeogenesis occurs. In 2013, Griffett *et al.* (55) described a molecule termed SR9238 (an ester derivative of GSK2033) as a liver-selective LXR dual-inverse agonist and showed that SR9238 treatment protected diet-induced obese mice from hepatosteatosis by decreasing the expression of lipogenic genes in the liver. They confirmed that the expression of the LXR target gene *Abca1* in the periphery was not affected by SR9238 treatment (55).

In addition to promoting the development of diabetes, long-term GC use is a major cause of secondary osteoporosis and muscle wasting. It would be interesting to investigate whether either of these two side effects is mitigated by LXRβ antagonism *in vivo*. Future studies could also investigate the utility of LXRβ antagonists as monotherapy for the treatment of diseases associated with endogenous GC excess such as Cushing disease (56) or type 2 diabetes (57). Owing to the limited systemic exposure of GSK2033, the applicability of an LXRβ antagonist for these conditions awaits the generation of a compound with increased systemic exposure and a longer half-life.

At present, orally available, potent LXRβ isoform-specific antagonists have not yet been generated. The development of such small molecules has the potential to provide therapeutic benefit to the millions of patients currently taking oral GCs for the treatment of severe inflammatory diseases.

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Author contributions: R.P. and C.L.C. conceived and designed experiments, analyzed the data, prepared the figures, and wrote the report. R.P. and L.M. performed *in vitro* experiments. R.P. performed *in vivo* experiments. R.T. managed the mouse colony and performed experiments with R.P. A.O. developed the synthesis for GSK2033 for *in vitro* and *in vivo* studies. S.A. contributed to experimental design and expertise in proteomic analyses.

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