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 Lxrβ−/− 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 the promoter of target genes and binds to glucocorticoid receptor response elements (GREs) to modulate gene expression. The ability of the GR to mediate the expression of pro-inflammatory genes and to induce gluconeogenesis has been well established (1, 5–8).

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; IC50, 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; pPCR, 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 Lxrα−/− mice (16, 28).

Recently, we demonstrated that whole body Lxrβ−/− 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 thiglycollate 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′gtctgagagagtcgacgggtgctg3′ and reverse primer 5′cggggtacctcttaaactcttcg3′. 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)
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 1-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 evening, 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 assayed. Plasma glucose was measured using a glucose oxidase kit (Promega, Madison, WI). 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 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 (Froestwood, 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) 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; 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

Lxr−/− livers were 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 1700g 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 $2 \times 10^5$ 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 $\pm$ 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-LXR$\alpha$ and 40 nM for GAL4-LXR$\beta$) in cell-based assays. We confirmed this level of activity in HEK293 cells expressing full-length receptors ($IC_{50}$ of 310 nM for LXR$\alpha$ and 83 nM for LXR$\beta$) (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-LXR$\alpha/\beta$ without affecting the DEX-induced activity of GAL4-GR.

GSK2033 showed preferential selectivity for LXR$\beta$, with an $IC_{50}$ of 167 nM, compared with LXR$\alpha$, with an $IC_{50}$ of 1.7 $\mu$M. This difference in potencies was not anticipated to be sufficient to selectively inhibit LXR$\beta$ in cells; thus, our studies were performed using LXR isoform-specific knockout mice.

**LXR$\beta$ 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 LXR$\beta$, 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, $Lxr\alpha^{-/-}$ and $Lxr\beta^{-/-}$ mice. As expected, DEX treatment significantly increased the expression of $Pepck$, a key gluconeogenic gene, in WT and $Lxr\alpha^{-/-}$ 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 $Lxr\alpha^{-/-}$ (from 15.7-fold to 3.2-fold) hepatocytes. In agreement with our previous studies, DEX treatment did not increase $Pepck$ expression in $Lxr\beta^{-/-}$ primary hepatocytes, and this remained unchanged with cotreatment of GSK2033 [Fig. 2(b)]. As expected, the LXR target gene, $Tat$, was significantly induced by DEX in WT (20.8-fold), $Lxr\alpha^{-/-}$ (12.7-fold), and $Lxr\beta^{-/-}$ (9.7-fold) hepatocytes and unchanged with GSK2033 cotreatment [Fig. 2(b)].

As expected, the LXR target gene $Streb1c$ was induced by T0901317 in hepatocytes from WT (37.7-fold), $Lxr\alpha^{-/-}$ (3.7-fold) and $Lxr\beta^{-/-}$ (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 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, Lxra−/−, 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, Lxra−/−, 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.
diminish DEX-induced Pepck expression in Lxrα−/− 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 Lxrα−/− 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 Lxrα−/− 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 Lxrα−/− 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 Lxrα−/− 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. Lxrα−/− 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 LXRα 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 PEPC 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 Lxrα−/− 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
nuclear GR translocation was significantly diminished in mice treated with DEX plus GSK2033 compared with DEX alone in *Lxrα*/*β* [Fig. 3(d) and 3(e)] and WT [Supplemental Fig. 3(c)] 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*/*β* 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.
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 Lxrβ−/− 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, Lxrα−/−, and Lxrβ−/− mice. As expected, DEX potently decreased LPS-induced...
expression of the proinflammatory genes, *Il1b* and *Il6*, regardless of genotype. Importantly, DEX-mediated *Il1b* 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 *Il1b* 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,
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 *Il1b* 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 in primary macrophages, it did not antagonize the anti-inflammatory actions of GCs in this system.

To assess the *in vivo* consequence of LXRβ antagonism with respect to GC-mediated effects on the immune system, we measured the spleen weight and liver expression of immune-responsive genes in *Lxrα2−/−* 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 LXRβ 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 LXRβ 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 (*Il1b*, *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 LXRβ antagonism achieves this effect in mice treated with DEX. The role for LXRβ in this context is surprising because LXRβ 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. 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 co-administration 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 proteome 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.
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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