Inverse Agonist of Nuclear Receptor ERRγ Mediates Antidiabetic Effect Through Inhibition of Hepatic Gluconeogenesis

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Type 2 diabetes mellitus (T2DM) is a progressive metabolic disorder with diverse pathological manifestations and is often associated with abnormal regulation of hepatic glucose production. Many nuclear receptors known to control the hepatic gluconeogenic program are potential targets for the treatment of T2DM and its complications. Nevertheless, the therapeutic potential of the estrogen-related receptor γ (ERRγ) in T2DM remains unknown. In this study, we show that the nuclear receptor ERRγ is a major contributor to hyperglycemia under diabetic conditions by controlling hepatic glucose production. Hepatic ERRγ expression induced by fasting and diabetic conditions resulted in elevated levels of gluconeogenic gene expression and blood glucose in wild-type mice. Conversely, ablation of hepatic ERRγ gene expression reduced the expression of gluconeogenic genes and normalized blood glucose levels in mouse models of T2DM: *db/db* and diet-induced obesity (DIO) mice. In addition, a hyperinsulinemic-euglycemic clamp study and long-term studies of the antidiabetic effects of GSK5182, the ERRγ-specific inverse agonist, in *db/db* and DIO mice demonstrated that GSK5182 normalizes hyperglycemia mainly through inhibition of hepatic glucose production. Our findings suggest that the ability of GSK5182 to control hepatic glucose production can be used as a novel therapeutic approach for the treatment of T2DM.
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Type 2 diabetes mellitus (T2DM), characterized by the presence of hyperglycemia, is a complex and progressive metabolic disorder with diverse pathological manifestations (1). Metformin (1,1-dimethylbiguanide hydrochloride), a member of the biguanide class of drugs, is an attractive therapeutic agent for the treatment of T2DM patients because it ameliorates cardiovascular mortality and hyperglycemia (2). Although metformin activates AMP-activated protein kinase and inhibits hepatic gluconeogenesis, the precise molecular mechanisms of metformin action on hepatic glucose metabolism have not been fully clarified.

Estrogen-related receptors (ERRs) belong to the NR3B group of the nuclear receptor superfamily, which includes the ERR subfamilies ERRα, ERRβ, and ERRγ. The newest member of the ERR subfamily, ERRγ, which was identified by cDNA library screening, interacts with the steroid receptor coactivator-2 (SRC-2) and the small heterodimer partner (SHP) (3,4). ERRγ is primarily expressed in heart, brain, kidney, pancreas, and liver and is induced during fasting in murine liver (3,5,6). The transcriptional activity of nuclear receptor ERRγ, which is constitutively active without natural ligands, depends on the interaction with nuclear receptor coactivators, such as SRC-2 and peroxisome proliferator–activated receptor (PPAR) γ coactivator 1α (PGC-1α), and nuclear receptor corepressors such as SHP and SMILE (SMall heterodimer partner Interacting LEucine zipper protein) (7–11). In hepatocytes, ERRγ regulates the expression of pyruvate dehydrogenase kinase 4 (PDK4) and leads to decreased oxidation of pyruvate to acetyl-CoA by phosphorylating pyruvate dehydrogenase complex (12). In addition, we previously reported that hepatic ERRγ contributes to impaired insulin signaling through the activation of diacylglycerol-mediated protein kinase ε and that ERRγ expression by cAMP signaling during fasting leads to induction of hepatic gluconeogenesis (13,14). These findings suggest that the use of a selective ligand to target the nuclear receptor ERRγ could be beneficial for the treatment of T2DM.

Several synthetic ligands reportedly repress the transcriptional activity of the ERRs by promoting or disrupting ERR-coactivator interactions (7). Diethylstilbestrol, a synthetic estrogen analog, represses the transcriptional activity of all ERRs, whereas 4-hydroxy tamoxifen (4-OHT), a selective estrogen-receptor modulator, inhibits the transcriptional activity of ERRβ and ERRγ but not ERRα (15). Although these synthetic compounds might be useful for studying the roles of ERRs, they can perturb the activity of

ERRs and other nuclear receptors, including estrogen receptors. GSK5182 (4-[(1Z)-1-{4-[2-(dimethylamino)ethoxy] phenyl}-5-hydroxy-2-phenylpent-1-en-1-yl]phenol), a 4-OHT analog, is a highly selective inverse agonist of ERRγ and does not interact with any other nuclear receptors, including ERRα or ERα, due to its additional noncovalent interactions with Y326 and N346 at the active site of ERRγ (13,16). Indeed, we demonstrated that GSK5182 directly and specifically inhibits the transcriptional activity of ERRγ in a PGC-1α–dependent manner and lowers hyperglycemia in *db/db* mice (13). However, the therapeutic efficacy of GSK5182 in the treatment of T2DM in vivo has not been fully elucidated. In this study, we examined the antidiabetic potential of GSK5182 in mouse models of T2DM.

RESEARCH DESIGN AND METHODS

Chemicals. GSK5182 was synthesized as previously described (13,17). GSK5182 was used in HCl salt form, dissolved in sterile-filtered 30% polyethylene glycol (PEG)-400 aqueous solution, and used at a concentration of 40 mg/kg for in vivo experiments. Metformin (1,1-dimethylbiguanide hydrochloride; Sigma-Aldrich, St. Louis, MO) was dissolved in solvent, as recommended by the manufacturer.

Recombinant adenovirus. Adenoviruses (Ads) expressing unspecific short hairpin (sh)RNA, shERRγ, control green fluorescent protein, and ERRγ were described previously (13). All viruses were purified by using CsCl or the Adeno-X Maxi Purification Kit (Clontech, Mountain View, CA).

Cell culture and transient transfection assay. Human embryonic kidney 293T cells were maintained as previously described (13). Transient transfection was performed using SuperFect (Qiagen, Hilden, Germany), according to the manufacturer's instructions. After 3–4 h of transfection, the medium was changed with DMEM containing 10% charcoal-stripped FBS. The cells were treated with GSK5182 (10 μmol/L), dexamethasone (100 nmol/L), rosiglitazone (100 nmol/L), fenofibrate (50 μmol/L), T0901317 (10 μmol/L), or T3 (100 nmol/L) for 24 h. The cells were harvested 48 h after transfection, and luciferase activity was measured and normalized to β-galactosidase activity.

Culture of primary hepatocytes. Primary hepatocytes were isolated from male Sprague-Dawley (SD) rats (weight 180–300 g) by collagenase perfusion (13) and cultured in medium 199 (Cellgro). After 3–6 h of culture, the attached cells were infected with the indicated Ads for 48 h before cell harvest and treated with GSK5182 (10 μmol/L) at 24 h before cell harvest.

Western blot analysis. Whole-cell extracts were prepared using RIPA buffer (Elpis-Biotech). Proteins from whole-cell lysates were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with monoclonal anti-ERRγ antibodies (R&D Systems, Tokyo, Japan). Immunoreactive proteins were visualized using an Amersham ECL kit (GE Healthcare, Piscataway, NJ), according to the manufacturer's instructions.

Animal experiments. Male C57BL6/J, *ob/ob* and *db/db* mice (7–12 weeks old; Charles River Laboratories) were maintained in a 12 h/12 h light/dark cycle and fed ad libitum. Blood glucose levels were measured at 4 h in fasting mice at day 6 after a tail-vein injection of Ad-ERRγ and at 17 h in fasting *db/db* mice at day 5 or at 17 h in fasting diet-induced obesity (DIO) mice at day 6 after Ad-shERRγ tail-vein injection. Mouse tail-vein injections of Ads were performed as previously described (18). Streptozotocin (STZ)-induced type 1 diabetic mice (treated with daily intraperitoneal injections of STZ [50 mg/kg body weight] dissolved in 10 mmol/L sodium citrate buffer for 5 days at 6–8 weeks old), *ob/ob*, *db/db*, and DIO mouse models and C57BL/6J mice fed a high-fat diet for 12 weeks (60 kcal % fat, D12492; Research Diets) were used to check hepatic ERRγ expression. To assess the short-term effects and to compare long-term antidiabetic and toxic effects of metformin (40 mg/kg/day) in *db/db* and DIO mice, GSK5182 (40 mg/kg/day) was injected intraperitoneally for 30 days in *db/db* mice and for 25 days in DIO mice. Blood glucose levels were monitored after 14 h of fasting.

Mice were killed at 30 days (*db/db* mice) or 26 days (DIO mice) after GSK5182 treatment. Plasma was collected from *db/db* and DIO mice, and aspartate aminotransferase (AST), alanine aminotransferase (ALT), blood urea nitrogen (BUN), creatinine (CRE), and creatine kinase (CK) levels were determined using an automatic blood chemistry analyzer (Hitachi7150, Japan). To examine fat accumulation in the livers of GSK5182-treated *db/db* and DIO mice, paraffin and frozen sections of liver tissue were prepared, stained with hematoxylin and eosin and with Oil-Red O, and observed by light microscope. All experiments were conducted according to the guidelines of the Sungkyunkwan University School of Medicine Institutional Animal Care and Use Committee.

In vivo imaging. C57BL/6J mice were infected with Ad-Pck1 WT-Luc (−2,371/+73) or Ad-Pck1 ERREmut-Luc via tail-vein injections. At 3 days after injection, mice were fasted for 16 h and given an intraperitoneal injection of GSK5182 (40 mg/kg) for 4 h. Mice were imaged using the IVIS 100 imaging system (Xenogen), as described previously (19).

Glucose and pyruvate tolerance test. C57BL/6J or DIO mice fasted for 4 or 17 h, respectively, were injected intraperitoneally with 1 g/kg glucose or 1 g/kg pyruvate. Blood glucose was measured in tail-vein blood using a One-Touch automatic glucose monitor (LifeScan Ltd., Milpitas, CA).

Glucose output assay. Primary rat hepatocytes were seeded and cultured for 24 h, and the medium was replaced with Krebs-Ringer buffer (115 mmol/L NaCl, 5.9 mmol/L KCl, 1.2 mol/L $MgCl_2$, 1.2 mmol/L NaH_2PO_4, 2.5 mmol/L $CaCl_2$, and 25 mmol/L $NaHCO_3$, pH 7.4), supplemented with 10 mmol/L lactate and 1 mmol/L pyruvate. The cells were treated with 10 μmol/L GSK5182 for 24 h. The glucose level in the media was measured using a QuantiChrom glucose assay kit (Bioassay Systems, Hayward, CA).

Measurement of metabolites. Blood triglycerides and insulin were measured using colorimetric assay kits (Wako Pure Chemical, Ltd., Osaka, Japan) and an Mouse Insulin ELISA kit (Shibayagi Co., Ltd., Ishihara, Japan), respectively.

Hyperinsulinemic-euglycemic clamp study. A hyperinsulinemic-euglycemic clamp study was performed in DIO mice, as previously described (20). Vehicle or GSK5182 (40 mg/kg/day) was injected intraperitoneally 5 days before the hyperinsulinemic-euglycemic clamp studies. After an overnight fast, [3-^{3}H]glucose (ARC, St. Louis, MO) was infused for 2 h to measure the basal glucose turnover. Subsequently, the hyperinsulinemic-euglycemic clamp was performed for 140 min with a primed/continuous infusion of human insulin (126 pmol/kg prime, 18 pmol/kg/min infusion; Eli Lilly, Indianapolis, IN) while plasma glucose was maintained at basal concentrations (~6.7 mmol/L). To measure insulin-stimulated whole-body glucose fluxes, [3-^{3}H]glucose was infused at a rate of 0.1 μCi/min throughout the clamps. Rates of basal and insulin-stimulated whole-body glucose fluxes and tissue glucose uptake were determined, as previously described (21).

Pharmacokinetic study of GSK5182. GSK5182 was administrated by intraperitoneal injection (10, 40, and 80 mg/kg) in SD male rats (n = 3). The drug was prepared as a solution (5 mg GSK5182 as an HCl salt in 2 mL PEG-400, saline, and DMSO at 40:55:5, v:v:v %). Blood samples were taken at 10 and 30 min and at 1, 2, 4, 6, 8, 12, and 24 h postinjection. After the purification of plasma by centrifugation, the concentration of GSK5182 was analyzed using an API 5000 LC/MS/MS system (AB SCIEX, Foster City, CA), a reverse-phase column (Hypersil GOLD, 50 × 2.1 mm, Thermo Scientific, Waltham, MA), and a photodiode array detector using electron spray ionization. Imipramine was used as an internal standard. Pharmacokinetic parameters were obtained after the analysis of plasma concentration-time plots with WinNonlin software (Pharsight, St. Louis, MO).

Quantitative PCR. Total RNA from primary hepatocytes or liver was extracted using an RNeasy Mini Kit (Qiagen). cDNA generated by Superscript II enzyme (Invitrogen) was analyzed by quantitative PCR using a SYBR green PCR kit and an TP800 Thermal Cycler DICE Real Time system (Takara). All data were normalized to ribosomal L32 expression.

Statistical analyses. All values are expressed as means ± SEM. The significance between mean values was evaluated by a two-tailed unpaired Student t test.

RESULTS

Hepatic ERRγ expression is induced by fasting and diabetic conditions. To extend our finding of the role of ERRγ in regulating gluconeogenesis in primary hepatocytes (13), we measured the expression levels of ERRs in mouse liver under fasting and refeeding conditions. As expected, the hepatic *ERRγ* mRNA and protein levels were significantly increased by fasting and reduced by refeeding in wild-type mice. The expression of hepatic gluconeogenic genes such as *Pck1*, *G6pc*, and *PGC-1α* was regulated in a similar fashion under these conditions (Fig. 1*A* and *B*). In addition, the hepatic expression of ERRγ and gluconeogenic genes was consistently increased in mouse models of type 1 diabetes mellitus (STZ-induced) and T2DM (*ob/ob*, *db/db*, or DIO mice; Fig. 1*C–F*). Consistent with mRNA levels, basal hepatic ERRγ protein levels were also higher in *ob/ob* and *db/db* mice than in wild-type mice (Fig. 1*D*). Interestingly, the hepatic expression of *ERRα* and *ERRβ* was not significantly changed by fasting or refeeding in wild-type mice and in the mouse models of T2DM (Fig. 1*A* and *F*).

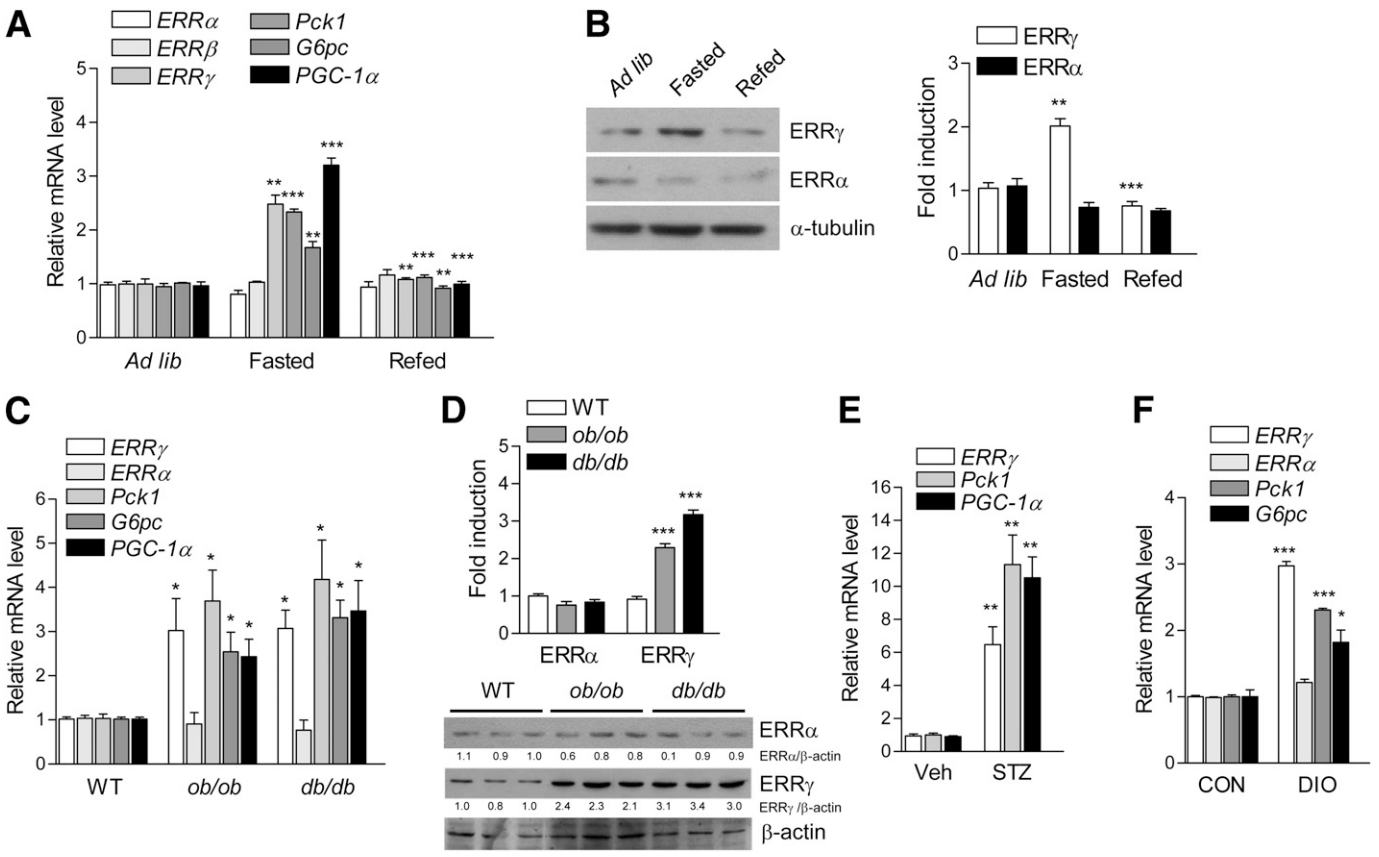

FIG. 1. Hepatic ERRγ gene expression during fasting and diabetic condition. *A*: Expression of hepatic *ERRγ* in response to fasting and refeeding in wild-type mice. Wild-type mice (n = 6) were fed ad libitum, fasted for 14 h, or fasted for 14 h and then refed for 6 h. *B*: Western blot analysis shows hepatic ERRα and ERRγ protein levels (*left*) and quantitation of ERRα and ERRγ protein levels (*right*). *C–F*: Hepatic expression of ERRs is shown in mouse models of diabetes. *C*: Quantitative PCR analysis of total RNA from the livers of wild-type, *ob/ob*, and *db/db* mice (n = 6). *D*: Graphic representation (*top*, ERRα or ERRγ/β-actin) and Western blot analysis (*bottom*) showing ERRα and ERRγ protein levels in livers of wild type, *ob/ob*, and *db/db* mice. Numbers on the blot show relative fold induction of ERRα or ERRγ protein level normalized to β-actin. *E*: Quantitative PCR analysis of total RNA from the livers of control mice administered vehicle (Veh) and STZ-treated mice (n = 3). *F*: Quantitative-PCR analysis of total RNA from the livers of wild-type and DIO (n = 3–5) mice. The error bars show ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001 by two-tailed Student t test. CON, control.

Moreover, the expression of all ERRs was not significantly altered in skeletal muscle during fasting and refeeding (Supplementary Fig. 1).

Hepatic ERRγ gene expression causes hyperglycemia through induction of gluconeogenic gene expression. We next hypothesized that alteration in ERRγ expression would affect hepatic glucose metabolism in vivo. To examine the hypothesis, we injected wild-type mice with an Ad overexpressing ERRγ and confirmed enhanced hepatic ERRγ protein levels (Fig. 2*A*). As expected, forced expression of ERRγ led to a significant induction of hepatic gluconeogenic gene expression and fasting hyperglycemia in wild-type mice, without significant changes in the plasma levels of AST, ALT, insulin, triglyceride, or nonesterified fatty acid (Fig. 2*B* and *C* and Supplementary Fig. 2*A–D*). Moreover, glucose excursion during an intraperitoneal glucose tolerance test (IPGTT) was significantly higher in Ad-ERRγ–injected mice than in control mice, and the blood glucose levels remained elevated for 2 h (Fig. 2*D*). These results raised the possibility that an increase in the transcriptional potential of ERRγ during diabetes may contribute to hyperglycemia in these settings. To test this hypothesis, we injected *db/db* mice with an Ad expressing a shRNA against ERRγ and confirmed a marked reduction in hepatic ERRγ expression at the mRNA and protein levels, with no effect on the expression of *ERRα* or *ERRβ* (Fig. 3*A* and *B*).

As expected, knockdown of endogenous ERRγ in the liver elicited a marked decrease in hepatic gluconeogenic gene expression and fasting blood glucose levels in *db/db* mice (Fig. 3*B* and *C*). However, plasma AST, ALT, insulin, and triglyceride levels were unaltered, whereas total cholesterol levels were higher in Ad-shERRγ–injected *db/db* mice than in Ad–unspecific shRNA (Ad-US)-injected control groups (Supplementary Fig. 3*A–D*). Because the DIO mouse model is widely used in studies of the pathophysiology of impaired glucose tolerance in T2DM (22), we further investigated whether knockdown of endogenous ERRγ improves hyperglycemia and glucose tolerance in DIO mice. ERRγ-deficient DIO mice showed reduced blood glucose levels and improved glucose tolerance compared with the control group (Fig. 3*D* and *E*). Taken together, these results demonstrate that modulation of ERRγ expression significantly affects hepatic gluconeogenesis in vivo.

GSK5182 specifically inhibits the transcriptional activity of ERRγ. GSK5182 is a highly selective inverse agonist of ERRγ due to its additional noncovalent interactions with Y326 and N346 of ERRγ (13,16). Indeed, a transient transfection reporter assay showed that GSK5182 specifically inhibited the transcriptional activity of ERRγ

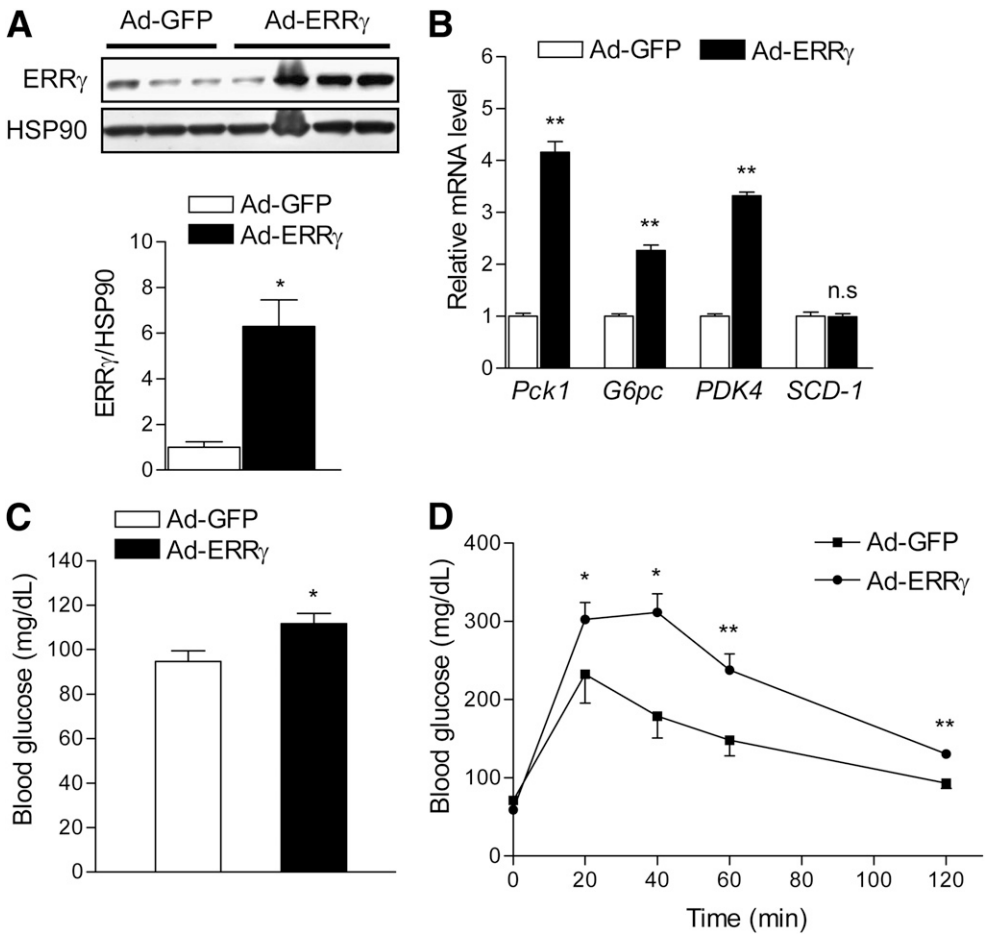

FIG. 2. ERRγ induces hepatic gluconeogenesis in vivo. *A–D*: Overexpression of ERRγ in liver results in the induction of gluconeogenic genes and fasting hyperglycemia. Ad-green fluorescent protein (GFP) or Ad-ERRγ was administered via tail-vein injection into wild-type mice (n = 3–4). *A*: Western blot analysis (*top*) and graphical representation (*bottom*) show ERRγ expression in liver. *B*: Quantitative PCR analysis of total RNA isolated from mouse liver at day 7 after injection. *C*: Four-hour fasting blood glucose levels in mice at day 6 after injection. *D*: Glucose tolerance test at day 5 after injection. Glucose was measured at the indicated times after 2 g/kg intraperitoneal glucose injection. Error bars show ± SEM. *P < 0.05, **P < 0.01 by two-tailed Student t test. HSP, heat shock protein; SCD-1, stearoyl-CoA desaturase-1.

but not of ERRα, ERRβ, or other ligand-dependent nuclear receptors, including thyroid hormone receptor α, liver X receptor α, glucocorticoid receptor, and PPARs (Supplementary Fig. 4*A–E*). On the basis of the ability of ERRγ to enhance the hepatic gluconeogenic program, we examined the effect of GSK5182 on gluconeogenic gene expression and glucose output in rat primary hepatocytes. GSK5182 markedly inhibited basal and Ad-ERRγ–mediated gluconeogenic gene expression and glucose production, but no such inhibition was observed in groups that received Ad-ERRγ Y326A, a mutant that cannot be targeted by GSK5182 (Fig. 4*A* and *B*). These results indicate that GSK5182 specifically inhibits the transcriptional activity of ERRγ, leading to decreased expression of gluconeogenic genes.

GSK5182 suppresses hepatic glucose production through inhibition of hepatic gluconeogenesis. Before examining the therapeutic potential of GSK5182 in insulin resistance and diabetes, we performed toxicity and pharmacokinetic studies of GSK5182 in vivo. In the acute toxicity study, ICR mice were orally administered GSK5182 (1,000 mg/kg, single treatment). Their body weights were monitored for 14 days, and there was no evidence of weight loss (Supplementary Fig. 5*A* and *B*). All animals were killed 14 days after treatment, and no toxic or adverse effects were found in postmortem examinations, confirming that the antidiabetic effect of GSK5182 can be evaluated in vivo in T2DM animal models (Supplementary Table 1).

In addition, GSK5182 at the dose of 40 mg/kg showed excellent pharmacokinetic behavior after intraperitoneal administration in SD male rats, with rapid absorption (time to peak concentration [T_{max}] of 30 min and maximum concentration [C_{max}] of 4.6 μmol/L) and stable plasma concentration of more than 0.22 μmol/L over 12 h (Fig. 4*C*). The dose-normalized C_{max}, area under the curve $(AUC)_{0\text{-}t}$, and $AUC_{0\text{-}\infty}$ were smaller for the 80 mg/kg dose than for the 10 and 40 mg/kg doses, indicating a saturation effect. These data suggest that the intraperitoneal 40 mg/kg dose of GSK5182 is within linear pharmacokinetic ranges and is sufficient for assuring a pharmacological activity in vivo. Hence, we chose the dose of 40 mg/kg for further in vivo evaluation.

On the basis of the pharmacokinetic profile of GSK5182, we performed in vivo imaging analysis to examine the inhibitory effect of GSK5182 on hepatic gluconeogenesis at the transcriptional level. Fasting-dependent induction of wild-type Pck1 promoter activity was significantly blunted in mice injected with GSK5182 (Fig. 4*D*, *left*). Ad-Pck1 ERREmut-Luc bearing ERRγ-binding site mutations also showed reduced promoter activity in mouse liver, and its activity was not altered by treatment with GSK5182 (Fig. 4*D*,

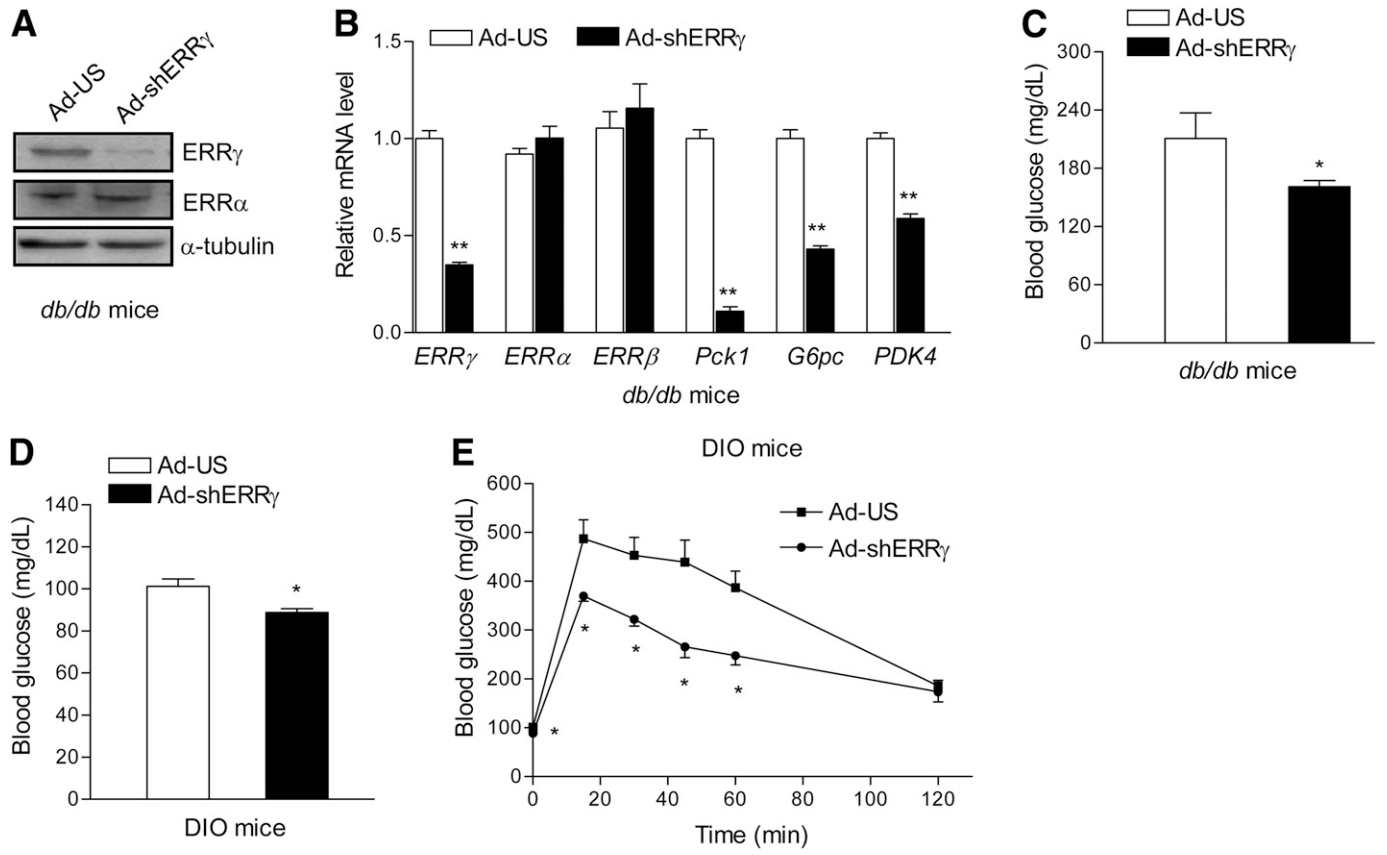

FIG. 3. Ablation of hepatic ERRγ improves hyperglycemia in mouse models of diabetes. *A–D*: Knockdown of ERRγ in liver in *db/db* mice leads to marked reduction of hepatic gluconeogenic genes and fasting blood glucose levels. Ad-US or Ad-shERRγ was injected via the tail vein into *db/db* mice (n = 4–6). *A*: Western blot analysis shows ERRα and ERRγ expression in liver. *B*: Quantitative PCR analysis of total RNA isolated from mouse liver after a 17-h fast. *C*: Blood glucose levels in mice fasted for 17 h at day 5 after injection. *D* and *E*: Fasting blood glucose levels were reduced in ERRγ-deficient DIO mice. Ad-US or Ad-shERRγ was injected via the tail vein into DIO mice (n = 4). *D*: Blood glucose levels in mice fasted for 17 h at day 5 after injection. *E*: Glucose tolerance test. Glucose was measured at the indicated times after glucose injection (1 g/kg i.p.) into DIO mice. Error bars show ± SEM. *P < 0.05, **P < 0.01 by two-tailed Student t test.

right). These results indicate that GSK5182 strongly suppresses the fasting-dependent stimulation of the gluconeogenic program in vivo through selective inhibition of the transcriptional activity of ERRγ. In addition, to examine the effect of GSK5182 on hepatic glucose production, we performed a pyruvate challenge test to monitor the changes in blood glucose levels in response to the administration of the gluconeogenic precursor pyruvate. GSK5182 strongly suppressed the pyruvate-dependent increase of blood glucose levels in DIO mice (Fig. 4*E*).

Finally, to further verify whether GSK5182 is directly responsible for the inhibition of hepatic glucose production in vivo, we performed hyperinsulinemic-euglycemic clamp studies in DIO mice. Indeed, hepatic glucose production was significantly decreased upon GSK5182 treatment at basal conditions and during the clamp period (Fig. 4*F* and *G*). Consistent with these changes, the whole-body glucose infusion rate and percentage inhibition of insulin-dependent hepatic glucose production were significantly increased in GSK5182-treated DIO mice (Fig. 4*H* and *I*). In addition, there is a small but significant increase in whole-body glucose disposal rates (Fig. 4*J*), suggesting that GSK5182 also affects insulin-sensitive peripheral tissues by enhancing their glucose uptake. However, GSK5182 treatment did not lead to significant changes in glucose uptake by skeletal muscle (Supplementary Fig. 6). These results show that the reduction of blood glucose levels by GSK5182 is largely due to decreased hepatic glucose production.

GSK5182 elicits antidiabetic effects in mouse models by negative regulation of the hepatic gluconeogenesis program. Finally, based on the inhibitory effect of GSK5182 on hepatic glucose production, we assessed the antidiabetic effect of GSK5182 in T2DM mouse models: *db/db* and DIO mice. We treated *db/db* mice with daily intraperitoneal injections (40 mg/kg) of GSK5182 or metformin, a gold standard antidiabetic drug, for 30 days. GSK5182 treatment dramatically reduced fasting blood glucose levels and gluconeogenic gene expression compared with the control group (Fig. 5*A* and *B*). On one hand, fasting blood glucose levels were restored to a nearly normal range in *db/db* mice (control, 446 ± 31 vs. GSK5182, 77 ± 3.9 mg/dL; fasting blood glucose of 77 mg/dL is within the range of normal fasting glucose levels [72–126 mg/dL] in humans) (Table 1 for glucose). Moreover, glucose excursion during an IPGTT was significantly attenuated in the GSK5182-treated group compared with the control group (Supplementary Fig. 7). On the other hand, blood glucose levels were higher in mice treated with metformin for 30 days (248 ± 33 mg/dL) than in mice treated with GSK5182 (77 ± 3.9 mg/kg), presumably because the concentration of metformin used was lower than that used in previous studies (23). Interestingly, GSK5182 treatment improved the liver toxicity caused by diabetes progression in *db/db*

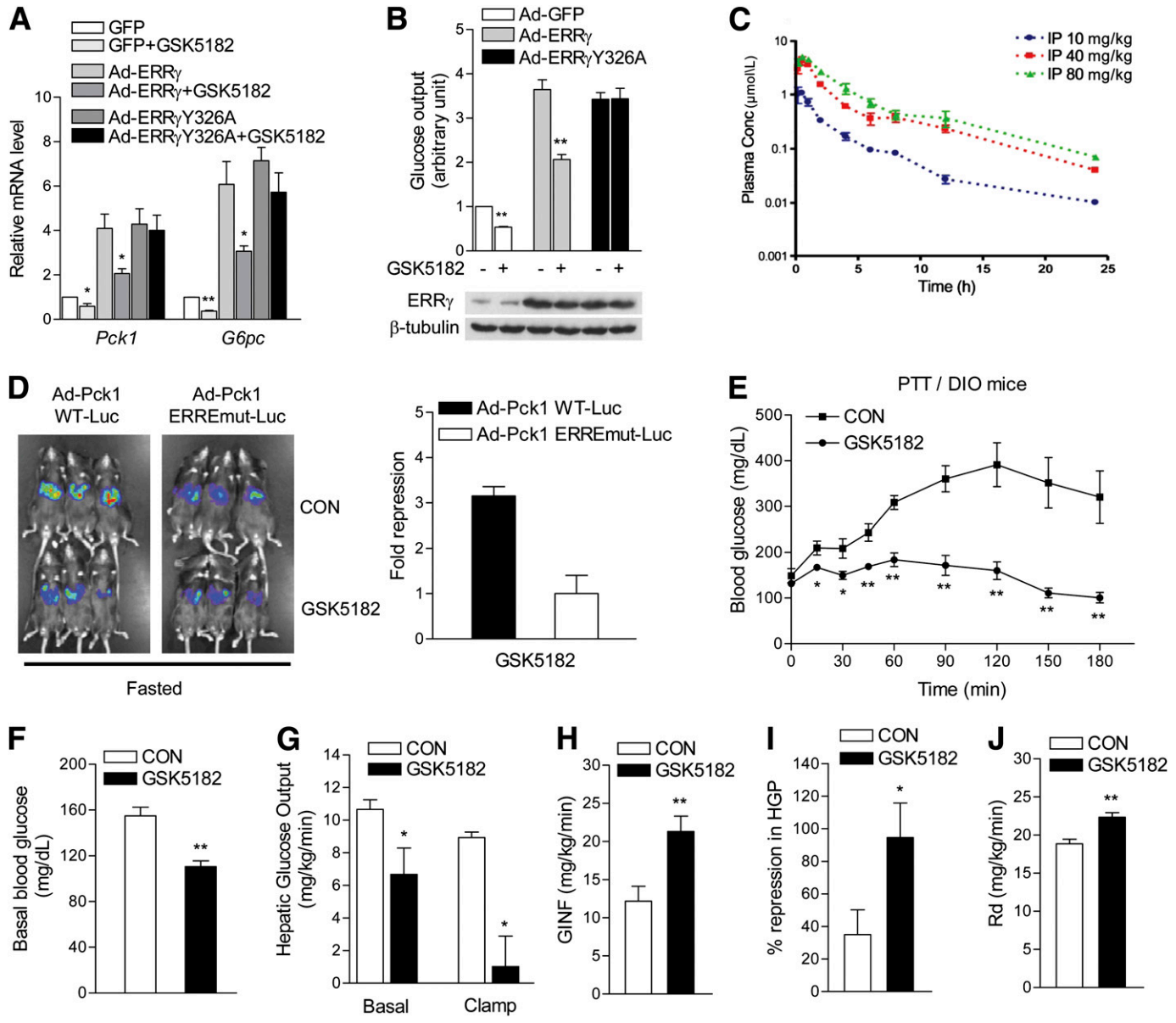

FIG. 4. GSK5182 lowers blood glucose levels through inhibition of hepatic gluconeogenesis in mice. GSK5182 specifically decreases ERRγ-induced gluconeogenic gene expression (*A*) and glucose production (*B*). *B*: Western blot analysis shows ERRγ and ERRγ Y326A expression (*bottom*). Primary rat hepatocytes were infected with the indicated Ads for the final 48 h before cell harvest and then treated with GSK5182 (10 μmol/L) for the final 24 h before cell harvest. *C*: Pharmacokinetic profile of GSK5182. Plasma concentration vs. time plots after intraperitoneal (IP) administration of GSK5182 (10, 40, or 80 mg/kg) for 14 days in SD male rats ($n = 3$). *D*: In vivo imaging of hepatic Ad-Pck1 WT-Luc and Ad-Pck1 ERREmut-Luc activity after GSK5182 treatment ($n = 3$) (*left*). Mice were treated with GSK5182 (40 mg/kg) for 4 h and then fasted for 16 h. Graphical analysis of luciferase activity after GSK5182 treatment (*right*). *E*: GSK5182 inhibits glucose production in response to pyruvate challenge. DIO mice ($n = 5$) fasted for 17 h after injection of vehicle or GSK5182 (40 mg/kg) were challenged with 1 g/kg pyruvate. *F*–*J*: Peripheral and hepatic insulin sensitivity was assessed by hyperinsulinemic-euglycemic clamps in DIO mice ($n = 9$–11). Vehicle or GSK5182 (40 mg/kg/day) was injected intraperitoneally for 5 days before hyperinsulinemic-euglycemic clamp studies. Basal blood glucose (*F*), basal and clamp hepatic glucose production (*G*), whole-body glucose infusion rate (GINF) (*H*), percentage inhibition of insulin-dependent hepatic glucose production (*I*), and rate of glucose disposal (Rd) (*J*). Error bars show ± SEM. *$P < 0.05$, **$P < 0.01$ by two-tailed Student *t* test. CON, control; PTT, pyruvate tolerance test.

mice, and there were no signs of kidney, muscle, or heart toxicity (Table 2 for BUN, CRE, and CK levels). Furthermore, GSK5182 treatment significantly decreased food intake and body weight and reduced hepatic fat accumulation as well as gonadal and inguinal fat mass (Fig. 5*C*–*F*).

To further investigate the antidiabetic effects of GSK5182 in DIO mice, we fed wild-type mice a high-fat diet for 15 weeks, which resulted in hyperglycemia (blood glucose levels after 14 h fasting were 134 ± 1 mg/dL). Vehicle, GSK5182 (40 mg/kg/day), or metformin (40 mg/kg/day) was injected intraperitoneally daily for 25 days, and blood glucose levels were measured every 5 days after 14 h of fasting. Administration of GSK5182 led to a significant reduction in fasting blood glucose levels beginning at day 5 and lasting until day 25 after treatment (Fig. 6*A* and *B*). Treatment with GSK5182 also reduced expression of gluconeogenic genes in the liver. Consistent with the results obtained in *db/db* mice, treatment with GSK5182 normalized hyperglycemia in DIO mice (control, 160 ± 9.2 mg/dL; GSK5182, 116 ± 2.0 mg/dL; metformin, 137 ± 5.2 mg/dL at day 25) (Table 2 for glucose).

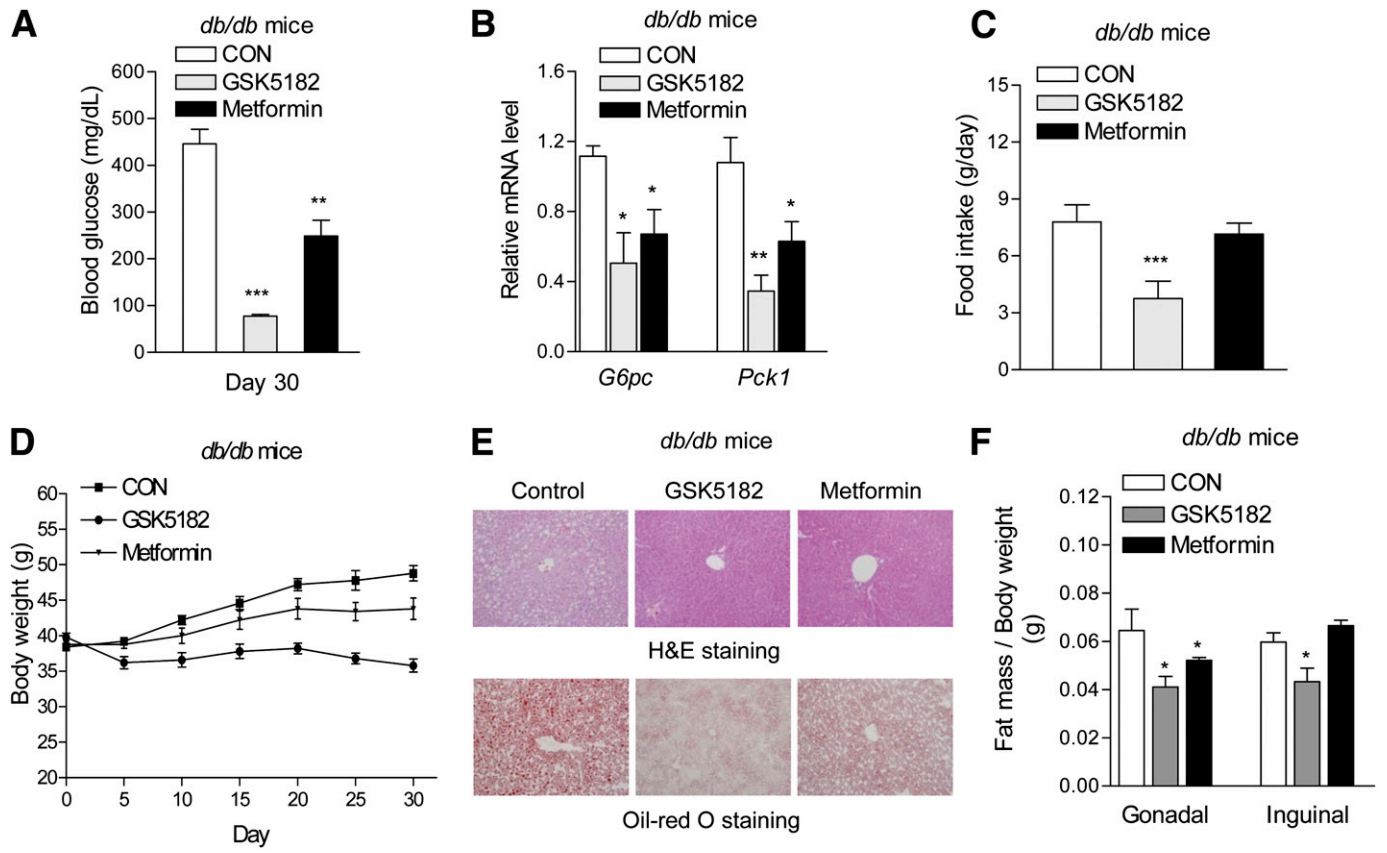

FIG. 5. GSK5182 treatment ameliorates hyperglycemia in *db/db* mice. *A–F*: GSK5182 lowers blood glucose through inhibition of hepatic gluconeogenic gene expression in *db/db* mice. Vehicle, GSK5182 (40 mg/kg/day), or metformin (40 mg/kg/day) was injected daily into *db/db* mice for 30 days ($n = 5$). *A*: Blood glucose was measured in tail-vein blood of *db/db* mice after a 14-h fast. *B*: Quantitative PCR analysis of gluconeogenic gene expression using total RNA isolated from mouse liver after a 14-h fast after the injection of vehicle, GSK5182, or metformin at day 30. *C*: Food intake. *D*: Body weight. *E*: Hematoxylin and eosin (H&E) and Oil-Red O staining of liver. *F*: Gonadal and inguinal fat mass. Error bars show ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by two-tailed Student *t* test. CON, control.

In addition, glucose excursion during an IPGTT was significantly lowered in the GSK5182-treated group compared with the control group (Supplementary Fig. 8*A*), and the reduction in glucose levels during an insulin tolerance test was greater in GSK5182-treated mice than in control mice (Supplementary Fig. 8*B*). Furthermore, liver toxicity caused by the progression of diabetic phenotypes in DIO mice was significantly improved by treatment with GSK5182, as evidenced by restoration of plasma AST and ALT levels to normal physiological ranges (AST, 9–60 IU/L; ALT, 5–40 IU/L; Table 2). There were no signs of kidney, muscle, or heart toxicity after treatment with GSK5182 or metformin (Table 2 for BUN, CRE, and CK levels). Food intake and body weight were decreased in GSK5182- and metformin-treated mice, which might be due to a decrease in gonadal and inguinal fat (Fig. 6*C*, *D*, and *F*).

Fat accumulation in the livers of DIO mice was dramatically reduced by GSK5182 treatment, consistent with the results obtained in GSK5182-treated *db/db* mice (Fig. 6*E*). However, GSK5182 treatment did not cause any significant changes in the activation of insulin-signaling proteins, such as IRS-1 and AKT, in skeletal muscle, liver, or gonadal fat (Supplementary Fig. 9*A*). Moreover, GSK5182 treatment did not affect the expression of ERRγ downstream target genes in skeletal muscle (Supplementary Fig. 9*B*). Taken together, these results suggest that GSK5182-mediated inactivation of ERRγ ameliorates the hyperglycemic phenotype in mice with T2DM largely by regulation of hepatic gluconeogenesis.

TABLE 1
Biochemical analysis of blood of *db/db* mice

	AST (IU/L) (range 9–60)	ALT (IU/L) (range 5–40)	BUN (mg/dL) (range >60)	CRE (mg/dL) (range 0.6–1.2)	CK (units/L) (range 60–400)	GLU (mg/dL) (range 72–126)
Control (vehicle)	139 ± 19	106 ± 9.6	22.8 ± 1.0	0.41 ± 0.04	1341 ± 445	446 ± 31
Metformin (40 mg/kg)	78.8 ± 11*	62.0 ± 11*	15.5 ± 2.1**	0.41 ± 0.03	374 ± 108	248 ± 33**
GSK5182 (40 mg/kg)	116 ± 35	83.0 ± 46	17.0 ± 2.0*	0.16 ± 0.03**	353 ± 51.5	77 ± 3.9***

GLU, glucose. Data are means ± SEM. The normal physiological ranges are provided in the column headings. After treatment with GSK5182 or metformin for 30 days, *db/db* mice were killed, and plasma samples were collected. AST, ALT, BUN, CRE, CK, and GLU levels were determined as described in RESEARCH DESIGN AND METHODS. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by two-tailed Student *t* test.

TABLE 2
Biochemical analysis of blood of DIO mice

	AST (IU/L) (range 9–60)	ALT (IU/L) (range 5–40)	BUN (mg/dL) (range >60)	CRE (mg/dL) (range 0.6–1.2)	CK (units/L) (range 60–400)	GLU (mg/dL) (range 72–126)
Control (vehicle)	132 ± 23	162 ± 37	26.6 ± 1.8	0.38 ± 0.02	160 ± 34.6	160 ± 9.2
Metformin (40 mg/kg)	69.8 ± 14	64.5 ± 24	25.5 ± 0.9	0.38 ± 0.03	419 ± 202	137 ± 5.2
GSK5182 (40 mg/kg)	55.5 ± 5.7**	19.5 ± 1.7***	26.6 ± 1.1	0.34 ± 0.02	284 ± 63.5	116 ± 2.0***

GLU, glucose. Data are means ± SEM. The normal physiological ranges are provided in the column headings. After treatment with GSK5182 or metformin for 26 days, DIO mice were killed, and plasma samples were collected. AST, ALT, BUN, CRE, and CK were determined as described in RESEARCH DESIGN AND METHODS. GLU was analyzed at day 25. $^{**}P < 0.01$, $^{***}P < 0.001$ by two-tailed Student t test.

DISCUSSION

In this study, we demonstrate that hepatic ERRγ, which is induced by fasting and diabetic conditions, promotes hepatic gluconeogenesis in vivo and that knockdown of ERRγ improves hyperglycemia in diabetic mice. In addition, the inverse agonist of ERRγ, GSK5182, lowers blood glucose levels through the inhibition of hepatic gluconeogenesis and thus ameliorates hyperglycemia in diabetic mice. Interestingly, hyperinsulinemic-euglycemic clamp studies showed that although GSK5182-treated DIO mice displayed a small but significant increase in whole-body glucose disposal rates, no significant changes occurred in glucose uptake by skeletal muscle. There was also no change in the levels of ERRγ expression or its downstream target genes. Furthermore, GSK5182-treated DIO mice did not display any significant change in insulin signaling in the liver, adipose tissues, or skeletal muscle, suggesting that GSK5182 inhibits ERRγ and facilitates glucose uptake in other tissues. Therefore, future studies are needed to investigate the additional effect of GSK5182 in peripheral tissues.

Long-term administration of GSK5182 in *db/db* and DIO mice decreased gonadal and inguinal fat mass (Fig. 5*F* and 6*F*). ERRγ gene expression is induced in inguinal adipose tissue in mice fed a high-fat diet and leads to enhanced differentiation of adipocytes through the regulation of adipogenic gene expression, including aP2, PPARγ, and PGC-1β (24). Therefore, it is tempting to speculate that GSK5182 inhibition of ERRγ suppresses the expression of adipogenic genes, which in turn inhibits adipocyte

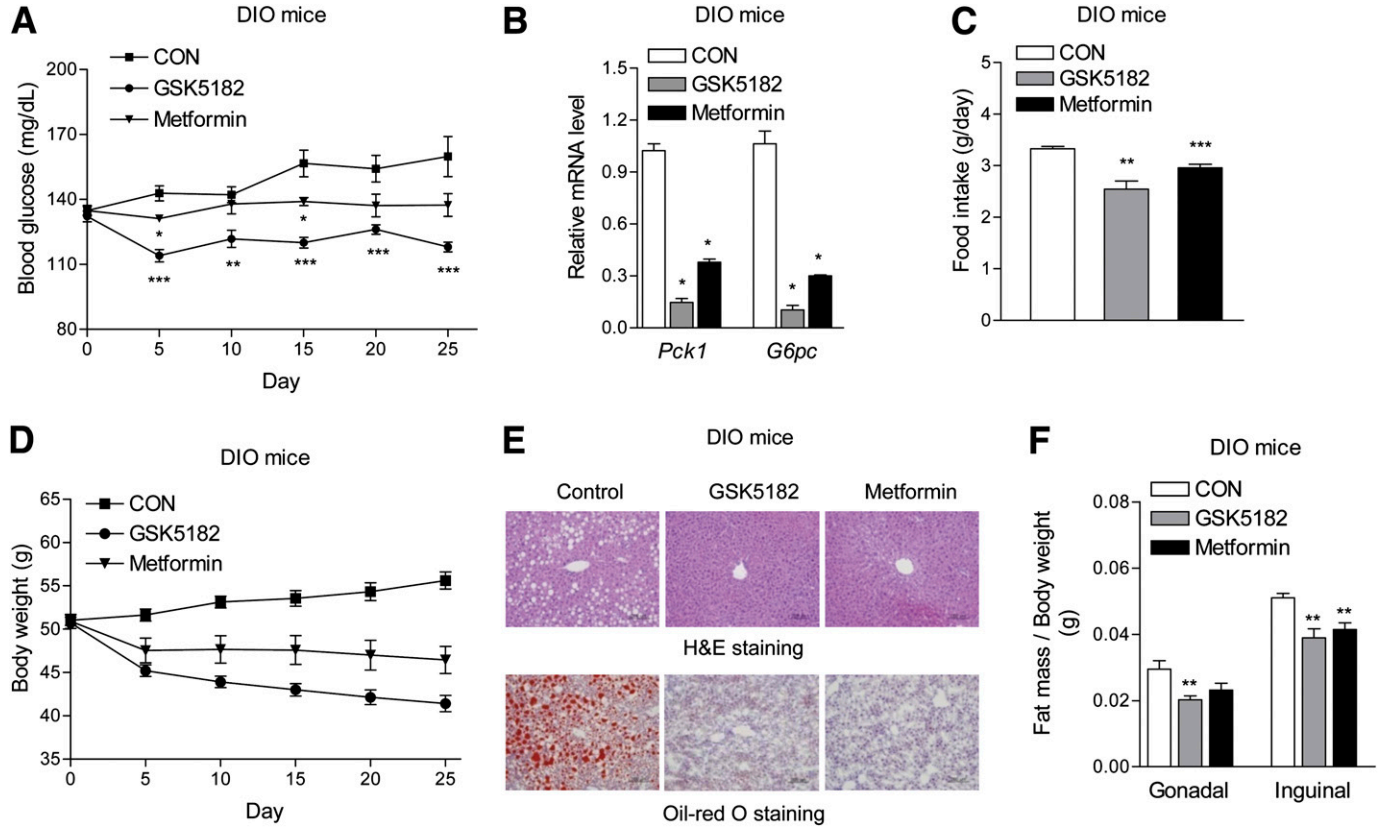

FIG. 6. GSK5182 mediates antidiabetic effect via inhibition of hepatic gluconeogenesis in DIO mice. *A–F*: Antidiabetic effects of GSK5182 and metformin in DIO mice. *A*: Blood glucose was measured after a 14-h fast in DIO mice injected with vehicle ($n = 5$), GSK5182 (40 mg/kg/day, $n = 9$), or metformin (40 mg/kg/day, $n = 5$) for 25 days. *B*: Quantitative PCR analysis is shown for gluconeogenic gene expression using liver RNA from mice after a 14-h fast and after the injection of vehicle, GSK5182, or metformin at day 26. *C*: Food intake. *D*: Body weight. *E*: Hematoxylin and eosin (H&E) and Oil-Red O staining of liver. *F*: Gonadal and inguinal fat mass. The error bars show ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by two-tailed Student t test. CON, control.

differentiation and leads to a reduction in fat mass. Moreover, GSK5182 treatment significantly decreased food intake and body weight in *db/db* and DIO mice (Fig. 5*C* and 6*C*), suggesting a role for ERRγ in the control of appetite. Extensive expression of ERRγ in the central nervous system (www.nursa.org/10.1621/datasets.02001) supports this hypothesis. Finally, the decrease in food intake per se likely diminishes fat mass in GSK5182-treated diabetic mice.

A previous study reported that ERRγ stimulates the expression of ERRα, which in turn activates the transcription of ERRα target genes upon association with ERRγ (6). However, we did not observe any significant change in the levels of ERRα in mice under fasting or diabetic conditions. In addition, ablation of endogenous ERRγ expression did not lead to significant changes in the expression of ERRα, ruling out potential compensatory roles of ERRα in the regulation of ERRγ target genes. These results prompted us to speculate that ERRα expression is not associated with the physiological nutrient status or pathological changes observed in T2DM and is not controlled by ERRγ under fasting conditions in the mouse liver. In support of this, we previously showed that ERRγ, its coactivator PGC-1α, and their targets, Pck1 and PDK4, are induced by the cAMP-signaling pathway during fasting (13). However, others have reported that ERRα acts as a repressor of the gluconeogenic gene Pck1 by directly inhibiting PGC-1α–induced Pck1 gene expression in the liver through inhibition of the recruitment of PGC-1α to the Pck1 promoter (25). In addition, these two isoforms recognize similar response elements (26), which might indicate the potential involvement of ERRα in the attenuation of ERRγ-dependent gluconeogenic gene expression by a negative feedback loop. Hence, we cannot exclude the role of ERRα as a negative regulator in ERRγ–PGC-1α–mediated regulation of hepatic gluconeogenesis. The molecular mechanisms involved in the expression and function of ERRα and ERRγ in the liver need to be further characterized.

In this study, GSK5182 treatment was associated with a significant antidiabetic effect, including normalized hyperglycemia in genetic and diet-induced diabetes. Furthermore, the liver toxicity and fatty liver caused by the progression of diabetic phenotypes in *db/db* and DIO mice were greatly improved by GSK5182 treatment, without signs of liver, kidney, muscle, or heart toxicity. This suggests that GSK5182 has a therapeutic potential for the treatment of complications associated with long-standing diabetes, as well as hyperglycemia, in T2DM. Finally, we propose that control of hepatic glucose production by GSK5182 can be used as a novel therapeutic approach for the treatment of hyperglycemia in T2DM.

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No potential conflicts of interest relevant to this article were reported.

D.-K.K., D.R., S.-H.K., and H.-S.C. designed and performed most of the experiments. D.-K.K., G.-T.G., Y.-H.K., J.P., C.-H.L., and S.-H.K. wrote the manuscript. M.K., T.S., and S.B.P. synthesized GSK5182 and performed the study of the pharmacokinetic profile of GSK5182. Y.-N.K., S.S.K., and C.S.C. performed the hyperinsulinemic-euglycemic clamp study. I.-K.L., C.S.C., S.B.P., C.-H.L., S.-H.K., and H.-S.C. contributed to discussion, review, and editing of the manuscript. H.-S.C. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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