Inhibition of 11 β -HSD1 by LG13 improves glucose metabolism in type 2 diabetic mice

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Abstract

11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1) controls the production of active glucocorticoid (GC) and has been proposed as a new target for the treatment of type 2 diabetes. We have previously reported that a natural product, curcumin, exhibited moderate inhibition and selectivity on 11 β -HSD1. By analyzing the models of protein, microsome, cells and GCs-induced mice *in vitro* and *in vivo*, this study presented a novel curcumin analog, LG13, as a potent selective 11 β -HSD1 inhibitor. *In vivo*, Type 2 diabetic mice were treated with LG13 for 42 days to assess the pharmacological benefits of 11 β -HSD1 inhibitor on hepatic glucose metabolism. *In vitro* studies revealed that LG13 selectively inhibited 11 β -HSD1 with IC₅₀ values at nanomolar level and high selectivity over 11 β -HSD2. Targeting 11 β -HSD1, LG13 could inhibit prednisone-induced adverse changes in mice, but had no effects on dexamethasone-induced ones. Further, the 11 β -HSD1 inhibitors also suppressed 11 β -HSD1 and GR expression, indicating a possible positive feedback system in the 11 β -HSD1/GR cycle. In type 2 diabetic mice induced by high fat diet plus low-dosage STZ injection, oral administration with LG13 for 6 weeks significantly decreased fasting blood glucose, hepatic glucose metabolism, structural disorders, and lipid deposits. LG13 exhibited better pharmacological effects *in vivo* than insulin sensitizer pioglitazone and potential 11 β -HSD1 inhibitor PF-915275. These pharmacological and mechanistic insights on LG13 also provide us novel agents, leading structures, and strategy for the development of 11 β -HSD1 inhibitors treating metabolic syndromes.

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Introduction

Glucocorticoid (GC) hormones are important metabolic regulators. Recent investigations have indicated that GC excess in tissues such as liver and adipose tissue contributes to the development of metabolic syndrome,

whose characteristics include central obesity, insulin resistance, atherogenic dyslipidemia, and hypertension (Masuzaki *et al.* 2001, Morton *et al.* 2005, Walker 2006, Macfarlane *et al.* 2008). 11 β -hydroxysteroid

dehydrogenase (11 β -HSD) type 1, which catalyses the conversion of active GCs (cortisol in humans and corticosterone in rodents) from their inactive 11-keto metabolites (cortisone in humans and 11-dehydrocorticosterone in rodents), can amplify the local level and activity of GCs (Masuzaki *et al.* 2001, Nixon *et al.* 2012, Chapman *et al.* 2013). On the other hand, 11 β -HSD2, as another isoform of 11 β -HSDs, catalyzes the opposite reaction, converting corticosterone to 11-dehydrocorticosterone in rodents (Chapman *et al.* 2013). Given that 11 β -HSD1 is abundantly expressed in metabolically important tissues which become resistant to insulin action in type 2 diabetes, such as adipose, muscle, and liver tissue (Chapman *et al.* 2013), inhibition of 11 β -HSD1 offers the ability to restore the metabolic action of insulin in these tissues. Furthermore, the lowering of intracellular GC concentration through a variety of means, including the administration of small molecular 11 β -HSD1 inhibitors, significantly attenuates the signs and symptoms of disease in animal models and in patients with diabetes/metabolic syndrome (Tahrani *et al.* 2011, Nixon *et al.* 2012, Harno *et al.* 2013a). In recent decades, a number of small molecule inhibitors of 11 β -HSD1 have been discovered, and several molecules have been evaluated in clinical trials.

Several small molecule inhibitors of 11 β -HSD1 were discovered in natural products, including glycyrrhetic acid, carbenoxolone, gossypol and estradiol (Cohen 2005, Zhang *et al.* 2009, Prasad Sakamuri *et al.* 2012). However, these natural products often showed low selectivity in the inhibition of 11 β -HSD1 over 11 β -HSD2. Thus, the development of synthetic structural analogs of natural products via rational drug design may be one approach for overcoming this low selectivity by finding new 11 β -HSD1-targeting candidates. Recently, RS Ge's group reported that curcumin, the main active component of turmeric isolated from the plant *Curcuma Longa*, exhibited potent inhibition of human and rat 11 β -HSD1 in intact cells, with a Two- to fivefold selectivity compared to 11 β -HSD2 (Hu *et al.* 2013). Our lab has long been engaged in curcumin-based drug design and discovery. We have designed and synthesized a series of mono-carbonyl curcumin analogues, which exhibited promising pharmacokinetic profiles (Liang *et al.* 2009a). As a part of a research collaboration, RS Ge's group also screened a series of curcumin analogs provided by our lab and found that some compounds specifically inhibited 11 β -HSD1 activity with much greater potency (with the IC₅₀s in nanomolar range) and much higher selectivity (with >100-fold more than 11 β -HSD2) than curcumin (Lin *et al.* 2013). Our previous studies reported the 11 β -HSD1-inhibitory

activity of 40 curcumin analogs, indicating that curcumin analogs may become novel therapeutic agents targeting 11 β -HSD1 for the treatment of metabolic syndrome.

Among our curcumin analogs which have been tested for 11 β -HSD1 inhibition, (2E,6E)-2,6-bis(2-(trifluoromethyl)benzylidene)cyclohexanone (LG13, Fig. 1A), shows the strongest activity *in vitro* and it also has a high selectivity compared to 11 β -HSD2. The aim of the present study was to examine the cellular target of LG13 and its pharmacological effects in type 2 diabetic mice. We also investigated that specific inhibition of 11 β -HSD1 is a promising therapeutic strategy for type 2 diabetes and metabolic syndrome.

Materials and methods

Reagents

Glucose, dexamethasone, prednisone, and the GC receptor (GR) selective inhibitor, mifepristone were purchased from Sigma. Corticosterone, 11-dehydrocorticosterone, NADP⁺, and NADPH were bought from Aladdin (Shanghai, China). Compound LG13 was synthesized in our lab using the method described in a previous publication (Liang *et al.* 2009b). Before being used for biological experiments, LG13 was recrystallized from CHCl₃/EtOH, and then HPLC was used to determine its purity (99.31%). The structures of curcumin and LG13 are shown in Fig. 1A. LG13 was dissolved in DMSO for *in vitro* experiments and was in 1% CMC-Na for *in vivo* experiments. Antibodies anti-11 β -HSD1 (sc-20175, dilution 1:400), anti-GR (sc-376426, dilution 1:500), and anti-AKT (sc-377457, dilution 1:300) were purchased from Santa Cruz Biotechnology; antibodies anti-pAKT (4060S, dilution 1:200) and anti-GAPDH (5174S, dilution 1:1000) were from Cell Signaling Technology (Danvers, MA, USA). The 11 β -HSD1 selective inhibitor PF-915275 was purchased from Tocris Bioscience (Abingdon, UK). Recombined human 11 β -HSD1 protein was produced by our lab and characterized by western blot and enzyme activity assay. Biochemical index analysis kits for total cholesterol (Tch), triglyceride (TG), LDL-c, alanine transaminase (ALT), and aspartate amino-transferase (AST) were purchased from Jiancheng Biotechnology Company (Nanjing, China).

Cell culture

Human hepatic cell line HL-7702 was obtained from the Institute of Biochemistry and Cell Biology, CAS (Shanghai, China). Cells were maintained in RPMI 1640

Figure 1

LG13 exhibited selective inhibition on 11 β -HSD1 enzyme activity. (A) Chemical structure of LG13. (B) 11 β -HSD1 inhibition of LG13 *in vitro*. Potency of LG13 against 11 β -HSD1 and 11 β -HSD2 enzymatic activities was determined as described in 'Materials and methods' section, and the IC₅₀

supplemented with 10% heat-inactivated FBS in humidified 37 °C and 5% CO₂ condition. Chinese hammary ovary cell line with overexpression of *HSD11B1* gene (CHO-*hs-HSD11B1* cells) was a gift from Prof. Renshan Ge (Population Council, New York, NY, USA) and cultured in DMEM medium supplemented with 10% heat-inactivated FBS in humidified 37 °C and 5% CO₂ condition.

11 β -HSD1/2 activity assay in cells and microsomes

Isolation of rat Leydig cells, preparation of rat testis microsomes and rat kidney microsomes, and the determination of 11 β -HSD1/2 activity were performed using the methods described previously (Hu *et al.* 2013).

Recombinant 11 β -HSD enzyme activity assay using HPLC method

The inhibitory effect of LG13 on 11 β -HSD1 reductase was measured using recombinant human 11 β -HSD1 protein through HPLC method. Briefly, 10 μ l recombinant 11 β -HSD1 protein (0.6139 mg protein/ml) was added to

was determined from more than ten determinations for each enzyme. (C) The inhibition of LG13 on recombinant 11 β -HSD1 reductase was analyzed via HPLC method (D, E, and F). The predicted binding mode of LG13 to 11 β -HSD1 was analyzed by computer-assisted molecular docking.

250 μ l buffer (100 mM KCl, 20 mM NaCl, 20 mM MOPS, pH 7.4) containing NADPH (1 mM) and 11-dehydrocorticosterone (11-dehydroCORT, 10 μ M) with or without various concentrations of LG13. The reactants were incubated at 37 °C for 20 min. After extraction with dichloromethane and evaporation, corticosterone (CORT) and 11-dehydroCORT concentrations were measured by HPLC (water: acetonitrile = 69–59: 31–41, v/v %) with u.v. detection. The inhibition rates were calculated using the concentration ratios of CORT and 11-dehydroCORT in tubes with or without LG13.

Molecular modeling

Tripes molecular modeling package Sybyl-x.v1.1.083 (Tripos, St Louis, MO, USA) was employed for the docking study. The crystal structure of 11 β -HSD1 was cited from the Protein Data Bank (PDB No. 2IRW), and residues around the ligand in this structure at a radius of 5 Å were isolated for construction of the docking grids. During the docking calculations, the ligand-binding groove on the proteins was kept rigid, whereas all torsible bonds of

ligands were set free to allow flexible docking to produce more than 200 structures. The ligand–receptor binding energy was set to be approximately the sum of the van der Waals and electrostatic interaction energies. After an initial evaluation of the orientation and scoring, a grid-based minimization was carried out for the ligand to locate the nearest local energy minimum within the receptor binding site. Final docked conformations were clustered within the tolerance of 1 Å root-mean-square deviation.

Animals

Male *C57BL/6J* mice weighing 18–22 g were obtained from the Animal Centre of Wenzhou Medical University (Wenzhou, China). Animals were housed at 25 °C with a 12 h light:12 h darkness cycle and fed with a standard rodent diet and water. The animals were acclimatized to the laboratory for at least 10 days before use. Protocols involving the use of animals were approved by the Wenzhou Medical University Animal Policy and Welfare Committee (Approval Document NO. wyd2014-0018).

Effect of LG13 on prednisone- or dexamethasone-treated mice

Male *C57BL/6J* mice (8 weeks old) were randomly assigned to five groups based on body weight ($n=8$ in each group). The experimental groups and respective treatments were as follows: i) control (vehicle: 1% CMC-Na, orally); ii) prednisone acetate (3 mg/kg per day, i.p.); iii) prednisone acetate (3 mg/kg per day, i.p.) plus LG13 (5 mg/kg per day, orally); iv) dexamethasone (2.5 mg/kg per day, i.p.); and v) dexamethasone (2.5 mg/kg per day, i.p.) plus LG13 (5 mg/kg per day, orally). Prednisone or dexamethasone was administered i.p. once daily for 15 days to induce metabolic disorders. LG13 was administered by oral gavage once daily at the same time as the prednisone or dexamethasone treatment. After 15 days of treatment, blood glucose level was determined in mice to investigate the effect of LG13 on prednisone- or dexamethasone-induced hyperglycemia. At the time of death, livers were harvested for 11 β -HSD1 determination by western blot and real-time quantitative PCR.

Effect of LG13 on type 2 diabetic mice

The diabetic mouse model was developed using a high fat diet plus a low dose of streptozotocin, similar to that described in previous studies (Shao *et al.* 2014). Male *C57BL/6J* mice were randomly divided into two groups:

control group (Ctrl) and high fat diet (HFD) plus streptozotocin injection group. In detail, the control group was fed standard chow, and the others were given HFD with 40% fat content (Slac Laboratory Animal, Shanghai, China). Following 2 months of dietary intervention, the HFD group was injected intraperitoneally with a low dose of streptozotocin (50 mg/kg). After 1 week, mice with a fasting blood glucose level > 14 mmol/l were considered to be diabetic. Diabetic mice ($n=40$) were assigned to five groups ($n=8$ in each group) and subjected to gavage treatment once per day with vehicle (1% CMC-Na), PF-915275 (5 mg/kg), pioglitazone (5 mg/kg), or LG13 (1 or 5 mg/kg), for 42 days. These mice continued to be fed their respective diets until the end of the study. The blood glucose levels of the mice were measured via blood drops obtained by clipping the tail of the mice using a ONE TOUCH BASIC plus Glucose Monitor (Lifescan, Milpitas, CA, USA). The blood glucose levels of these animals were recorded every week. An oral glucose tolerance test (OGTT) was carried out in mice deprived of food for 12 h (2 g/kg glucose administered by gavage) at day 41 of the treatment (Levy *et al.* 2014, Moreno *et al.* 2014). After the OGTT determination, the mice were killed under ether anesthesia at day 42 after drug treatment. Blood samples were collected via the retro-orbital sinus for the determination of a series of biochemical indexes. The livers were dissected, weighed, immediately frozen in liquid nitrogen and/or embedded in 4% paraformaldehyde for subsequent analysis. TG, TCh, LDL, ALT, and AST in serum or livers were analyzed using the respective kits with a colorimetric method (Hitachi 7020 Automatic Analyzer, Japan).

Liver histopathology

Liver tissues were fixed in 4% paraformaldehyde and embedded in paraffin. The paraffin sections (5 μ m) were stained with hematoxylin and eosin (H&E). To estimate the extent of damage, the slides were observed under a light microscope (200 $\times$, Olympus).

Quantitative real-time PCR

Cells or liver tissues (50–100 mg) were homogenized in TRIZOL (Invitrogen) for the extraction of RNA according to the manufacturer's protocol. Both reverse transcription and quantitative PCR were carried out using a two-step M-MLV Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen). The Eppendorf realplex4 detection system (Eppendorf, Hamburg, Germany) was used for

quantitative-PCR analysis. The primers of tested genes were synthesized from Invitrogen. The primer sequences used are listed in Table S1, see section on [supplementary data](#) given at the end of this article. The amount of each gene was determined and normalized to the amount of β -actin.

Western immunoblot analysis

Fifty micrograms of total protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to a nitrocellulose membrane. Each membrane was pre-incubated for 1 h at room temperature in Tris-buffered saline, pH 7.6, containing 0.05% tween-20 and 5% non-fat milk, and incubated with specific antibodies. Immunoreactive bands were then detected by incubating the membrane with secondary antibodies conjugated to HRP, and visualized using ECL reagents (Bio-Rad). The protein amounts were analyzed using Image J analysis Software version 1.38e (NIH, Bethesda, MD, USA) and normalized with their respective control.

Statistical analysis

Each *in vitro* experiment was performed in a group size of $n > 3$ independent samples. Representative images from three independent experiments were shown. The results are presented as mean $\pm$ s.d. The statistical significance of difference between groups was obtained by student's *t*-test or ANOVA multiple comparisons in GraphPad Pro (GraphPad, San Diego, CA, USA). Multiple comparisons between the groups were performed using *post-hoc* S-N-K method. $P < 0.05$ was considered as indication of statistical significance.

Results

LG13 strong inhibition on 11 β -HSD1 enzyme activity

As described in our previous papers (Hu *et al.* 2013, Lin *et al.* 2013), a series of curcumin analogs have been tested for their inhibitory potency against 11 β -HSD1/2 enzyme activity. The results of LG13 are shown in Fig. 1B with curcumin as a comparison. Testis and leydig cells have extremely high expression and activity of 11 β -HSD1. Using rat leydig cells and microsomes from rat testis, which have high 11 β -HSD1 activities, we found that LG13 showed strong inhibitory effects against rat 11 β -HSD1,

with IC₅₀ values of 0.10 and 0.18 μ M respectively. We then constructed CHO-*hs-HSD11B1* cell line, which over-expresses the *HSD11B1* gene and exhibits 11 β -HSD1 activity. Low IC₅₀ values were also observed when LG13 inhibited 11 β -HSD1 activity in CHO cells transfected with *HSD11B1*. LG13 showed greater potency (8.2- to 57-fold) for the inhibition of 11 β -HSD1 activity than curcumin. 11 β -HSD2, on the other hand, acts as a dehydrogenase and catalyzes the opposite reaction, down-regulating the activities of GC in kidney and colons. Thus, the rat kidney microsomes were prepared and used for the 11 β -HSD2 activity assay. More importantly, compound LG13 did not inhibit rat 11 β -HSD2 at all at 100 μ M, while curcumin showed slight selectivity for 11 β -HSD2 (Fig. 1B). To explore whether LG13 inhibits 11 β -HSD1 activity in a microsome-free system, recombinant human 11 β -HSD1 protein was incubated with or without LG13 and the enzymatic activity (conversion of 11-dehydrocorticosterone to corticosterone) was tested using HPLC. As shown in Fig. 1C, LG13 also dose-dependently decreased the enzymatic activity of recombinant 11 β -HSD1 protein, indicating that LG13 might directly bind 11 β -HSD1 and inhibited its activity.

To explain the mode of interaction between LG13 and 11 β -HSD1, a molecular docking simulation was performed based on the X-ray crystal structure of the 11 β -HSD1 complex with a synthetic and selective inhibitor (PDB structure No. 2IRW). In the initial crystal structure, hydrogen bonds provided strong interactions between the ligand and the protein. As shown in Fig. 1D, the carbonyl group of LG13 formed one hydrogen bond with Ser170 of 11 β -HSD1 without NADPH, and the CF₃ group of LG13 formed the second hydrogen bond with Ala172. In addition, Tyr177 might be involved in hydrophobic contact with the benzene ring of LG13. However, the conformational shift induced by co-substrate NADPH binding removed the hydrogen bond interactions between LG13 and 11 β -HSD1, in which residues Leu126, Tyr183, Tyr177, and Val227 formed a hydrophobic pocket and were involved in the hydrophobic contacts with LG13 (Fig. 1D and E). The docking results also showed that LG13 forms hydrophobic contacts with NADP using respective benzene rings (Fig. 1F).

Inhibition of 11 β -HSD1 enzyme activity by LG13 led to the decrease in 11 β -HSD1 expression

Previous studies showed that GC which is active downstream of 11 β -HSD1 could increase 11 β -HSD1 expression as a positive feedback to the pathology

(Bujalska *et al.* 2006, Yang *et al.* 2009, Zhu *et al.* 2010, Wang *et al.* 2013). We then determined whether LG13 could inhibit 11 β -HSD1 expression by inhibiting 11 β -HSD1 activity. First, we showed that treatment of hepatic HL7702 cells with dexamethasone (DEX, an active GC) for 1 h significantly increased the protein expression of 11 β -HSD1 (Fig. 2A) and GR (Fig. 2B). Pretreatment with mifepristone (Mif), a GR antagonist, markedly reversed DEX-induced 11 β -HSD1 and GR expression. A previous study also found that incubation with a high concentration of glucose (HG) could increase the expression of 11 β -HSD1 (Fan *et al.* 2011). Thus, we investigated the effects of 11 β -HSD1 inhibitors on 11 β -HSD1 expression in the HG system. As shown in Fig. 2C and D, HG treatment for 90 min increased the expression of 11 β -HSD1 and GR, while the enzyme inhibitors including PF-915275 (PF), a well-known potent 11 β -HSD1 inhibitor, and LG13, significantly reduced HG-induced overexpression of 11 β -HSD1 itself as well as GR. LG13 also showed a greater inhibitory effect than PF (Fig. 2C and D). These data indicate that there is a positive feedback mechanism in the 11 β -HSD1/GR activity-expression pathway, and the down-regulation of 11 β -HSD1/GR expression by small molecule inhibitors may be used to investigate their effects on the activity of 11 β -HSD1.

LG13 inhibited prednisone-induced changes, but had no effect on DEX induction

The data previously shown have demonstrated that LG13 could directly bind and inhibit 11 β -HSD1 at the protein, microsome, and cell levels. Here, we further investigated

the beneficial effects of LG13 both *in vitro* and *in vivo*, which were mediated by its inhibition on 11 β -HSD1. DEX is a synthetic active GC directly binding and activating GR, whereas prednisone (PRE) is a synthetic cortisone analogue (inactive GC), which has little affinity for GR (Chapman *et al.* 2013). However, PRE can be catalyzed by liver 11 β -HSD1 to its active metabolite, prednisolone, which acts as an active GC. We detected the effects of LG13 on DEX- and PRE-treated cells and mice respectively.

As shown in Fig. 3A and B, stimulation with both DEX and PRE for 1 h significantly increased the expression of 11 β -HSD1 and GR protein in HL7702 cells. Pretreating with LG13 (5 and 10 μ M) for 2 h dose-dependently reduced PRE-induced 11 β -HSD1 expression, whereas no obvious inhibition was observed in DEX-treated cells. Similar results were observed at the mRNA level (Fig. 3C and D). We next confirmed the selective inhibition of LG13 in mice treated with DEX and PRE. Mice were intraperitoneally injected with DEX or PRE at the indicated dosage for 15 days, with or without oral administration of LG13 at 5 mg/kg. The dosages of DEX and PRE used here are decided in our preliminary experiments, in which we found that prednisone acetate (3 mg/kg per day, *i.p.*) and dexamethasone (2.5 mg/kg per day, *i.p.*) led to a comparable pathological induction in mice. Figure 4A shows that both DEX and PRE significantly increased 11 β -HSD1 protein expression in mice livers, while oral administration with LG13 inhibited PRE-induced 11 β -HSD1 expression by 99.6%. A similar result was observed in hepatic tissue, where LG13 was found to have no effect on the DEX-increased 11 β -HSD1 mRNA level (Fig. 4B). A previous study has also demonstrated that

Figure 2

Inhibition of 11 β -HSD1 enzyme activity led to the decrease in 11 β -HSD1 expression. (A and B). GR regulated the expression of 11 β -HSD1. Liver HL7702 cells were pretreated with vehicle or mifepristone (Mif, 10 μ M) for 1 h, then treated with 1 μ M DEX for another 1 h. Total protein was extracted and examined by western blotting analysis using specific antibodies. (C and D) PF-915275 and LG13 decreased HG-induced 11 β -HSD1 and GR expression. Liver HL7702 cells were pretreated with PF-915275

(10 μ M), or LG13 (5 or 10 μ M), or vehicle for 2 h, then stimulated with HG (30 mM) for 90 min. Total protein was extracted and examined by western blotting analysis. The column graphs show the normalized optical density from more than three independent experiments (*P < 0.05, **P < 0.01, ***P < 0.001 compared with HG). A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0268>.

Figure 3

LG13 inhibited prednisone-induced changes, but had no effect on dexamethasone's induction *in vitro*. Liver HL7702 cells were pretreated with LG13 (5, 10 μ M), or DMSO for 2 h, then stimulated with DEX (1 μ M) or PRE (1 μ M) for 24 h. The expression of 11 β -HSD1 and GR were detected by western blot (A and B) or quantitative real-time PCR (C and D) as described in 'Materials and methods' section. Bars represent the mean $\pm$ S.E.M. of more than three independent experiments (* P <0.05, ** P <0.01 compared with PRE). A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0268>.

GCs can induce insulin resistance and increase blood glucose levels (Macfarlane *et al.* 2008). As shown in Fig. 4C, induction by either DEX or PRE led to a 1.5-fold increase in fasting blood glucose level in mice (P <0.01), while treatment with LG13 only significantly attenuated PRE-induced hyperglycemia, not DEX-induction. This evidence showed LG13 inhibited the PRE-induced changes but had no effect on the downstream DEX's action, indicating that 11 β -HSD1 is the direct target of LG13 and mediates the biological effects of LG13 both *in vitro* and *in vivo*.

Administration of LG13 lowered fasting blood glucose, and improved glucose tolerance and hepatic glucose metabolism in type 2 diabetic mice

To validate the beneficial effects of LG13 *in vivo*, an experimental type 2 diabetic mouse model was established for analyzing the pharmacological functions of LG13. As Fig. 5A shows, LG13 administered by oral gavage for 6 weeks reduced fasting glucose concentrations to 53.8% of the diabetic mice, and these remained significantly lower (P <0.01) throughout the treatment period. The glucose-lowering effect of LG13 was dose-dependent and stronger than that of the positive controls of PF and pioglitazone (Piog) at the same dosage (5 mg/kg). The type 2 diabetic

mice showed glucose intolerance, as evaluated by OGTT, while glucose tolerance was improved by the treatment with LG13, PF, and Piog, at the dosage of 5 mg/kg (Fig. 5B). Plasma glucose tolerance levels, as determined by the area under curve (AUC) of the glucose concentration curve, were reduced by 15.2, 25.7, and 33.6% compared to vehicle control following administration of 5 mg/kg PF, Piog, and LG13 respectively (P <0.05, Fig. 5C).

The AKT/PI3K pathway plays an important role in the development of insulin resistance (Glass & Olefsky 2012). As shown in Fig. 5D, the phosphorylation of AKT (also known as protein kinase B) in DM was decreased, while administration of PF, Piog or LG13 all significantly prevented AKT dephosphorylation (P <0.05). It has been demonstrated that gene products including insulin receptor substrate (IRS)-1, glucose transporter (GLUT)-4, adiponectin, and fatty acid binding protein (FABP)-4 are involved in the development of hepatic insulin resistance (Becker *et al.* 2004, Tiikkainen *et al.* 2004, Subash-Babu *et al.* 2015). Therefore, these biochemical parameters in mice livers were detected by quantitative real-time PCR. As shown in Fig. 5E, F, G, and H, mice in the DM group had decreased hepatic gene expression of IRS-1, GLUT-4, and adiponectin, but increased FABP-4 mRNA levels. Treatment with LG13 dose-dependently reversed these changes in diabetic mice livers. Interestingly, PF and Piog showed different effects on the above changes compared to LG13

Figure 4

LG13 inhibited prednisone-induced changes, but had no effect on dexamethasone's induction *in vivo*. After 15 days of treatment, the mice were killed and liver tissues from each group were individually processed for protein and RNA extraction. Relative expression of 11 β -HSD1 in livers was detected by western blot (A) and quantitative real-time PCR (B). Before mice were killed, blood glucose level was determined (C) (n =8 in each group, * P <0.05 compared with PRE group). A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0268>.

Figure 5

Administration of LG13 decreased fasting blood glucose, and improved glucose tolerance and hepatic glucose metabolism in type 2 diabetic mice. PF-915275 (PF) and pioglitazone (Piog) at the dosage of 5 mg/kg were used as positive comparison. (A) The fasting blood glucose levels at day 0, 1, 7, 17, 27, 37, and 42 after LG13 administration were monitored ($n=8$). (B and C) Effect of compounds on OGTT ($n=8$). After the last treatment with compounds, mice were fasted overnight (12 h), followed by administrated with D-glucose (2 g/kg, p.o.). The blood glucose levels at 0, 15, 30, 60 and 120 min were detected (B), and the area under each curve was calculated respectively (C). The data in A–C were analyzed using

Repeated Measures ANOVA. (D) LG13 administration reversed the injured insulin signaling. At the time of death, mice livers were harvested and processed for protein extraction. The levels of p-AKT/AKT were determined by western blot analysis. (E, F, G, and H). Effect of LG13 on insulin resistance-related genes expression. At the time of death, mice livers were harvested and processed for RNA extraction. Relative gene expression of IRS-1 (E), adiponectin (F), GLUT-4 (G), and FABP-4 (H) were detected by quantitative real-time PCR ($n=8$, $*P<0.05$, $**P<0.01$, $***P<0.001$ compared with DM group). A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0268>.

Table 1 Effect of LG13 on biochemical parameters in serum and livers of diabetic mice

Parameters	Ctrl	DM	DM+PF (5 mg/kg)	DM+Piog (5 mg/kg)	DM+LG13 (1 mg/kg)	DM+LG13 (5 mg/kg)
Serum TG (mmol/l)	1.97 $\pm$ 0.32	3.51 $\pm$ 0.74	3.04 $\pm$ 1.04	2.07 $\pm$ 0.52**	1.88 $\pm$ 0.23**	1.58 $\pm$ 0.14***
Serum TCh (mmol/l)	0.55 $\pm$ 0.07	1.09 $\pm$ 0.05	0.90 $\pm$ 0.13*	0.97 $\pm$ 0.04**	0.95 $\pm$ 0.005**	0.84 $\pm$ 0.08**
Serum LDL-C (mmol/l)	0.52 $\pm$ 0.12	0.85 $\pm$ 0.09	0.47 $\pm$ 0.09**	0.45 $\pm$ 0.10***	0.56 $\pm$ 0.17*	0.46 $\pm$ 0.11***
Liver TG (mmol/l)	0.32 $\pm$ 0.07	0.51 $\pm$ 0.07	0.74 $\pm$ 0.17*	0.52 $\pm$ 0.15	0.59 $\pm$ 0.14	0.35 $\pm$ 0.07*
Liver ALT (U/l)	0.82 $\pm$ 0.12	1.02 $\pm$ 0.13	0.98 $\pm$ 0.19	0.79 $\pm$ 0.20	0.88 $\pm$ 0.06	0.76 $\pm$ 0.11**
Serum ALT (U/l)	2.83 $\pm$ 0.32	3.85 $\pm$ 0.17	2.38 $\pm$ 0.41***	3.58 $\pm$ 0.53	2.84 $\pm$ 0.75*	2.33 $\pm$ 0.49***
Serum AST (U/l)	23.9 $\pm$ 9.77	77.8 $\pm$ 14.7	28.5 $\pm$ 7.54***	31.2 $\pm$ 10.8**	18.3 $\pm$ 0.86***	10.2 $\pm$ 7.96***

TG, triglyceride; TCh, total cholesterol; ALT, alanine transaminase; AST, aspartate aminotransferase. All results were represented by mean $\pm$ s.d. ($n=8$, * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ compared with DM).

at the same dosage of 5 mg/kg. PF only reversed the changes of IRS-1 and FABP-4, while Piog only showed a significant influence on FABP-4 overexpression in diabetic livers. These results indicated that 11 β -HSD1 inhibitors effectively attenuated hyperglycemia and improved hepatic glucose metabolism in type 2 diabetic mice, and LG13 exhibited stronger pharmacological effects than PF and Piog. In addition, the fact that LG13 could affect AKT phosphorylation and IRS1/GLUT4 expression in diabetic mice indicates that the anti-diabetes effects of 11 β -HSD1 inhibitor may be associated with the attenuation of impaired early insulin signaling and the increase in IRS-1/GLUT4 expression which contribute to the basal glucose uptake.

Metabolic effects of the 11 β -HSD1 inhibitor LG13 in the livers of type 2 diabetic mice

Inhibition of 11 β -HSD1 has been shown to improve serum lipid profiles and alleviate fatty liver in mice with metabolic syndrome or type 2 diabetes (Nixon *et al.* 2012, Stewart & Tomlinson 2009). We next investigated

whether LG13 treatment could attenuate such pathological changes. In DM mice, there were significant increases in serum levels of TG, TCh, and LDL-c (Table 1). All of the tested drugs, including PF, Piog, and LG13, lowered the serum levels of these three indexes ($P<0.01$). LG13 showed a dose-dependent reduction which was greater than that observed with PF and Piog. LG13 at 5 mg/kg also exhibited the inhibition of DM-increased levels of hepatic TG, while no significant attenuation was observed in other treated groups (Table 1). TG levels were reduced by 55 and 31% in the serum and livers of LG13-treated mice respectively. When compared to controls, DM induced a significant increase in the ratio of liver weight to body weight, while PF and LG13 at 5 mg/kg reduced the DM-induced increase in liver weight after 42 days of treatment (Fig. 6A). Further, H&E analysis of liver sections demonstrated that DM-induced hepatic steatosis was substantially attenuated by oral administration of LG13 at 5 mg/kg for 6 weeks (Fig. 6B). The type 2 diabetic mice had increased hepatocellular ballooning degeneration, increased lipid content, inflammatory lesions, and fibrosis when compared to the control

Figure 6

Administration with LG13 attenuated the hepatic lipid deposition in type 2 diabetic mice. (A) LG13 administration lowered hepatic weight/body weight ratio ($n=8$, * $P<0.05$, ** $P<0.01$ compared with DM). (B) LG13

administration significantly attenuated hepatic steatosis. At the time of death, mouse liver tissues were processed for H&E staining as described in 'Materials and methods' section (200 $\times$ amplification).

mice and those diabetic animals received with 5 mg/kg LG13. This change was accompanied by a marked reduction in the serum and liver levels of the liver injury markers ALT and AST (Table 1). However, PF and Piog could not prevent the increase in ALT in diabetic mouse livers. Taken together, the new 11 β -HSD1 inhibitor LG13 showed more potent pharmacological effects on abnormal lipid metabolism and fatty liver injury than the positive controls, PF and Piog, at the same dosage.

Administration of LG13 inhibited the hepatic 11 β -HSD1/GR pathway in diabetic mice

At the time of death, mice livers were harvested for analyzing the protein and gene expression in the 11 β -HSD1/GR pathway. As shown in Fig. 7A, B, C, and D, diabetic mice expressed higher levels of 11 β -HSD1 and GR in liver tissues, while treatment with PF, Piog, or LG13 (1 or 5 mg/kg) significantly reduced the expression of 11 β -HSD1 and GR at both the protein and mRNA levels ($P < 0.05$). As expected, LG13 showed a dose-dependent

effect and stronger activity than PF and Piog. GCs are known to increase the expression of two key gluconeogenic enzymes: phosphoenolpyruvate carboxykinase (PEPCK) and catalytic subunit glucose-6-phosphatase (G6Pase), which play important roles in liver glucose homeostasis and insulin sensitivity (Lu *et al.* 2013). Figure 7E showed that LG13 dose-dependently reduced mRNA levels of PEPCK in livers from 6.42 in the DM group to 1.67 and 1.23 ($P < 0.01$) after the administration at 1 and 5 mg/kg dosage, respectively. The levels of G6Pase mRNA also decreased after LG13 treatment (Fig. 7F). Although similar results were observed in the PF- and Piog-treated groups, both showed relatively lower inhibition than LG13. These data implied that the anti-diabetic benefits of LG13 are accompanied by its inhibition of the hepatic 11 β -HSD1/GR pathway.

Discussion

Excess GC accumulation may result in several metabolic syndromes, such as insulin resistance, obesity, and type 2 diabetes, in both animals and humans (Walker 2006). 11 β -HSD1 is abundantly expressed in liver and adipose tissues, where it plays a key role in regulation of the local generation of active GCs (Masuzaki *et al.* 2001, Chapman *et al.* 2013). Increases in active GCs in liver tissues are closely related with the pathogenesis of metabolic syndromes. Overexpression of 11 β -HSD1 in the mouse liver caused active GC excess which induced metabolic disorders, while 11 β -HSD1 knockout mice were resistant to high fat diet-induced metabolic syndromes (Harno *et al.* 2013a,b, Paterson *et al.* 2004). Thus, pharmacological intervention with 11 β -HSD1 is a potential therapeutic method for preventing metabolic diseases. Courtney *et al.* (2008) reported the activity of PF-915275, a potent and small-molecular 11 β -HSD1-selective inhibitor exhibited improvement of hepatic insulin sensitivity and reduction of lipid deposition in type 2 diabetic patients. In addition, three 11 β -HSD1 selective inhibitors, AMG221, INCB13739, and DIO-902, are now being investigated in clinical trials (Schwartz *et al.* 2008, Tiwari 2010, Gibbs *et al.* 2011).

In previous studies, we firstly reported that curcumin potentially inhibits 11 β -HSD1 activity in intact cells (Hu *et al.* 2013). It exhibited a moderately selective inhibition on 11 β -HSD1, two- to fivefold that on 11 β -HSD2. Curcumin is a natural active product with multiple pharmacological functions (Asher & Spelman 2013). However, its clinical application has been significantly limited by its fast metabolism and poor bioavailability

Figure 7

Administration with LG13 inhibited hepatic 11 β -HSD1/GR pathway in diabetic mice. Western blot analysis was used to determine the protein expression of 11 β -HSD1 (A) and GR (B). Column figures showed the normalized optical density as percentage of the loading control GAPDH. In addition, relative gene expression of 11 β -HSD1 (C), GR (D), PEPCK (E), and G6Pase (F) were detected by quantitative real-time PCR respectively ($n = 8$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with DM). A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-14-0268>.

(Epstein *et al.* 2010). Our lab has designed and synthesized a series of structural analogs of curcumin in order to discover novel agents with stronger 11 β -HSD1 inhibition and higher selectivity (Liang *et al.* 2009a). Of these curcumin analogs, LG13, exhibited strong inhibitory effect and high selectivity against 11 β -HSD1 over 11 β -HSD2. LG13 is a novel and potent 11 β -HSD1 selective inhibitor. With PF-915275 as a positive control, the present study describes the action and mechanism of a novel selective 11 β -HSD1 inhibitor, LG13, on glucose homeostasis and hepatic insulin sensitivity in type 2 diabetic mice.

Inactive GCs, such as prednisone, can be catalyzed by 11 β -HSD1 into active metabolites, such as prednisolone, which induces hyperglycemia and metabolic syndromes (Courtney *et al.* 2008). Therefore, theoretically, the 11 β -HSD1 inhibitor cannot attenuate the active GC-induced pathological changes. A previous study reported that an 11 β -HSD1 inhibitor, emodin, could reverse prednisone-induced insulin resistance in obese mice, but had no effect on dexamethasone induction (Feng *et al.* 2010). Similar results were observed in the action of LG13. Our data reveal that LG13 only decreased prednisone-induced 11 β -HSD1 and GR overexpression, but had no significant effect on those induced by dexamethasone in both hepatic cells and mice models. Subsequently, oral administration of LG13 had little effect on dexamethasone-induced hyperglycemia, while it significantly decreased prednisone-induced hyperglycemia (Fig. 4C). However, the usage of discrepant doses of prednisone and dexamethasone may be a limitation of this study. The dosages used here are referred to our preliminary experiments, in which we found that prednisone acetate (3 mg/kg per day, i.p.) and dexamethasone (2.5 mg/kg per day, i.p.) led to a comparable pathological induction in mice. The dosage difference may be attributed to drug sensitivity, drug metabolism, and salt forming. Although they reached a comparable level in stimulating changes, the data in Fig. 4 showed slightly higher levels of 11 β -HSD1 protein and mRNA with dexamethasone than with prednisone treatment. The dosage difference did not influence the conclusion that LG13 inhibited prednisone but not dexamethasone. Therefore, these findings validate that LG13 specifically inhibits 11 β -HSD1 and functions by inhibiting 11 β -HSD1 *in vitro* and *in vivo*.

The phenotype of type 2 diabetes has been associated with increasing hepatic 11 β -HSD1 activity. Induction of hepatic 11 β -HSD1 activity could increase local active GC production, then directly act on the GR, which is a nuclear receptor regulating genes in development, metabolism,

and the immune response (Zhou & Cidlowski 2005, Snyder *et al.* 2011). Although 11 β -HSD1 expression also plays an important role in the development of metabolic syndromes, in recent years pharmacological interventions targeting 11 β -HSD1 have mainly focused on the inhibition of 11 β -HSD1 enzymatic activity. Several stimuli, including HG, TNF- α , and lipopolysaccharide (LPS) have been shown to stimulate the expression of 11 β -HSD1 in different cells (Ishii *et al.* 2007, Ignatova *et al.* 2009, Fan *et al.* 2011). It has been reported that TNF- α -induced 11 β -HSD1 expression was mediated by p38 MAPK, CCAAT/enhancer binding protein (C/EBP)- β , and NF- κ B pathways (Ignatova *et al.* 2009). Interestingly, several studies have demonstrated that active GCs have the ability to increase 11 β -HSD1 expression (Yang *et al.* 2009, Zhu *et al.* 2010). The regulation of GR transcriptional activity on 11 β -HSD1 expression is still unclear. In the present study, we found that dexamethasone could significantly induce 11 β -HSD1 and GR expression while either mifepristone or LG13 remarkably inhibited dexamethasone-induced 11 β -HSD1 and GR expression in hepatic cells (Fig. 2A and B). To our knowledge, this is the first time that 11 β -HSD1 inhibitors, such as PF-915275 and LG13, have been shown to not only inhibit 11 β -HSD1 activity, but also decrease HG- and hyperglycemia-induced 11 β -HSD1 and GR expression *in vitro* and *in vivo* (Figs 2C, 7A, and C). This finding reveals the relationship between 11 β -HSD1 activity and its own expression, and indicates a possible positive feedback system in the 11 β -HSD1/GR cycle.

Further, we found that oral administration of LG13 at 5 mg/kg for 6 weeks significantly decreased fasting blood glucose, hepatic glucose metabolism, histological disorders, and lipid deposition type 2 diabetic mice. Compared with pioglitazone and PF-915275, LG13 exhibited better pharmacological effects. Thus, the novel 11 β -HSD1 inhibitor, LG13, is a potential agent for preventing type 2 diabetes. In addition, we also noted that 11 β -HSD1 is expressed in other tissues, including adipocytes, muscle, and pancreatic island, which are also in relation to glucose metabolism and insulin resistance. Thus, 11 β -HSD1 in other tissues may be also in response to GL13 to regulate glucose metabolism. Here we only demonstrated the 11 β -HSD1/GR and pathological changes in diabetic mouse liver. Further study should be carried out to observe the effects of LG13 in other tissues. In summary, this work discovered a positive feedback system for 11 β -HSD1 expression and activity, and demonstrated a new 11 β -HSD1 inhibitor, LG13, is a potential agent for preventing type 2 diabetes and hepatic glucose metabolism.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-14-0268>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

L Z, R G, D L, G L and Y W conceived and designed the experiments. Y P, K P, and Z W performed the experiments. L Z, Y P, C T, and Y W analyzed the data. G L, Y P, and Y W wrote and reviewed the paper.

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