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A selective sphingomyelin synthase 2 inhibitor ameliorates diet induced insulin resistance *via* the IRS-1/Akt/GSK-3β signaling pathway

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Insulin resistance is a typical precursor and primary feature of type 2 diabetes mellitus (T2DM). Sphingomyelin (SM) is a kind of sphingolipid located in animal brain, liver, kidney and muscle. Sphingomyelin synthase 2 (SMS2) is the key enzyme in the synthesis of sphingomyelin, inhibition of which shows protective effects on cardiovascular and glucose metabolism. We used Ly93, a selective sphingomyelin synthase 2 inhibitor, to investigate the effect of SMS2 inhibitor on insulin resistance *in vitro* and *in vivo*. Our previous studies have shown that Ly93 is able to dose-dependently inhibit the SMS activity and attenuate the atherosclerotic lesions in apoE knock out mice. In this present study, we found that high fat diet (HFD) induced insulin-resistant C57BL/6 mice treated with Ly93 were more sensitive to insulin than untreated mice, and presented lower blood insulin levels and improved insulin tolerance. Furthermore, insulin signal pathway related protein levels were detected by western blot, which indicated that SMS2 inhibitor significantly upregulated the phosphorylation of IRS-1, Akt and GSK-3β, thus enhanced the insulin signaling. *In vitro*, Ly93 enhanced the phosphorylation of Akt in HepG2 cells, which was reversed by exogenous sphingomyelin. These results suggest that SMS2 inhibitor could ameliorate insulin resistance *via* regulating the insulin signaling. Our findings support that SMS2 is a potential target for insulin resistance.

1. Introduction

Diabetes mellitus (DM) is a common clinical metabolic disease, characterized by hyperglycemia. Modern high-calorie diet and bad habits have increased the incidence of diabetes (Deed et al. 2015; Wenger 2012). T2DM is the main type of diabetes, which is a long-term metabolic disorder characterized by high blood sugar and insulin resistance (Pandey et al. 2015). Insulin resistance is a typical precursor to the onset of T2DM, constantly linked with impaired insulin signaling (Riobo Servan 2013). Some researchers have revealed that insulin regulates glucose metabolism via the IRS-1/PI3K/Akt pathway (Kadowaki et al. 2012) and the downstream proteins such as GLUT-2, GLUT-4 and GSK-3β (Gao et al. 2017; Zhang et al. 2016).

SM is a type of sphingolipid located in and constitutes some microdomains of plasma membrane, synthesized at the endoplasmic reticulum (ER) and the Golgi by sphingomyelin synthases (SMSs) (Bienias et al. 2016). SM plays a crucial role at cell functions like cell death, proliferation and migration, and plasma SM level is considered to be an indicator to some human diseases like atherosclerosis (Taniguchi et al. 2014). SMSs, including SMS1, SMS2, are the key enzymes and involved in the last step of sphingomyelin de novo biosynthesis in vivo. SMSs can catalyze the conversion of ceramide to sphingomyelin—SMS1 in Golgi and SMS2 in plasma membrane, separately (Huitema et al. 2004). SMSs modulate sphingomyelin and other sphingolipids levels, including ceramide and diacylglycerol (DAG) (Chen et al. 2017).

SMS is considered to be related to many human diseases like atherosclerosis and diabetes from previous studies. Many studies have shown that the inhibition or gene deficiency of SMS can contribute to the attenuation of the atherosclerotic lesions and inflammation reduction in atherosclerosis model animals (Li et al. 2012). As for diabetes, it has been reported that the SMS gene deficiency can improve mouse insulin sensibility (Li et al. 2011; Mitsutake et al. 2011; Sugimoto et al. 2016). It is obvious, that SMS inhibitors have a potential to become effective therapeutic substances.

T2DM and cardiovascular disease share a large number of incentives, such as obesity and sedentary lifestyle, therefore the morbidity and mortality of atherosclerotic cardiovascular disease in individuals with T2DM is very high (Geiss et al. 2014). Beyond that, statins, the initial therapy for atherosclerosis, has been considered to elevate the risk of new onset diabetes and might impair adipocyte browning in some large-scale clinical trials and meta-analysis (Balaz et al. 2018; Casula et al. 2017; Thakker et al. 2016). In consideration of the strong correlation between diabetes and cardiovascular disease, a treatment that is beneficial for both these two disease areas (Yuan et al. 2013).

In this present study, we used a selective SMS2 inhibitor Ly93 (Fig. 1) to investigate the relationship between SMS2 and insulin resistance *in vitro* and *in vivo*. Ly93 is a potent SMS2 inhibitor with exhibits IC_{50} values of 91 nM and 133.9 μM against purified SMS2 and SMS1 respectively, which has been proven to be able to attenuate the development of atherosclerosis (Li et al. 2018). We hypothesized that Ly93 could ameliorate diet induced insulin resistance in C57BL/6 mice, and that this is associated with regulation of insulin signaling.

Abbreviations: T2DM, Type 2 diabetes mellitus; SM, sphingomyelin; SMS, sphingomyelin synthase; SMS2, sphingomyelin synthase 2; HFD, high fat diet; IC_{50}, 50% inhibiting concentration; ER, endoplasmic reticulum; DM, diabetes mellitus; DAG, diacylglycerol; ND, normal diet; FBG, fasting blood glucose; Fins, fasting blood insulin; HOMA-IR, homeostasis model assessment of insulin resistance; OGTT, oral glucose tolerance test; ITT, insulin tolerance test; DMEM, Dulbecco's modified Eagle's medium.

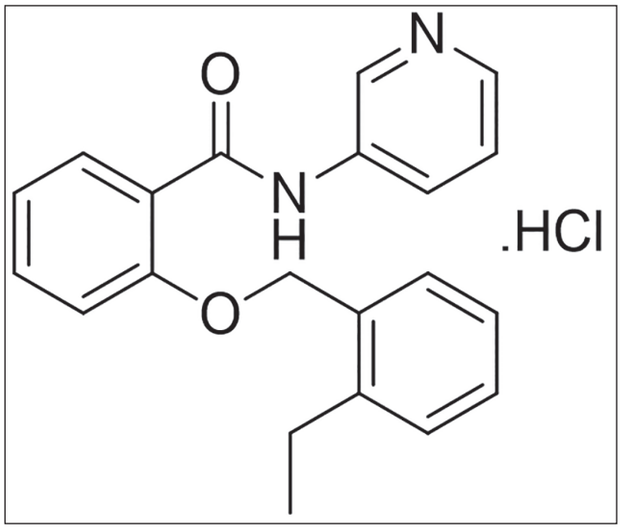

Fig. 1: Structure of Ly93.

2. Investigations and results

2.1. Effects of Ly93 on body weight, fasting blood glucose (FBG), fasting blood insulin (Fins) and homeostasis model assessment of insulin resistance (HOMA-IR) in mice

The body weight was evaluated every week during the 12 weeks treatment (Fig. 2A). At the last week, it was obvious that the body weights of mice in model group were higher than in control group ($P < 0.05$). Moreover, Ly93 mildly declined the body weights, but not significantly ($P = 0.08$).

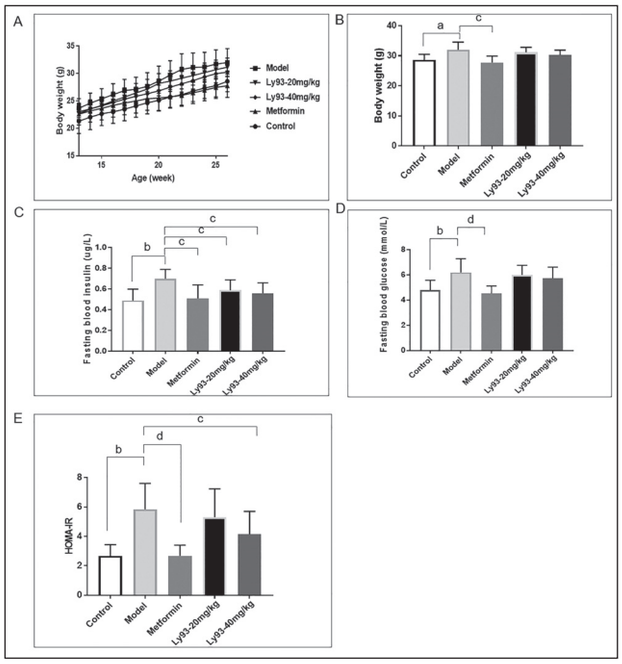

Fig. 2: Effects of Ly93 on body weight, fasting blood glucose (FBG), fasting blood insulin (Fins), homeostasis model assessment of insulin resistance (HOMA-IR) in C57BL/6 mice. (A) Body weight trend of mice. (B) Body weight of mice after Ly93 treatment for 12 weeks. (C) FBG of mice after Ly93 treatment for 12 weeks. (D) Fins of mice after Ly93 treatment for 12 weeks. (E) HOMA-IR of mice after Ly93 treatment for 12 weeks. The HOMA-IR was calculated as serum insulin (mmol/L) * (blood glucose (mmol/L)/22.5. Control, normal control group (ND, solvent for metformin and Ly93); Model, model group (HFD, solvent for metformin and Ly93); Metformin, metformin group (HFD, metformin 0.2 g/kg/day); Ly93-20 mg/kg, 20 mg/kg Ly93 group (HFD, Ly93 20 mg/kg/day); Ly93-40 mg/kg, 40 mg/kg Ly93 group (HFD, Ly93 40 mg/kg/day). Data are means±SD; n = 10 per group; a represents $P < 0.05$ vs control, b represents $P < 0.01$ vs control, c represents $P < 0.05$ vs model, d represents $P < 0.01$ vs model.

The FBG and Fins levels were measured after 12 weeks treatment. As shown in Fig. 2C, in the model group, the FBG concentrations were significantly increased after a HFD for 12 weeks compared to control group ($P < 0.05$). FBG concentrations of two Ly93 groups were similar with model groups. The Fins concentrations are shown in Fig. 2D. We found that the Fins concentrations were elevated significantly (38 %, $P < 0.05$) in the model group *versus* the control group. Whereas the Ly93-20 mg/kg group and the Ly93-40 mg/kg group both showed significant reduction of Fins (16 % each, $P < 0.05$) *versus* the model group. To further investigate the insulin resistance status of mice, the HOMA-IR index was calculated from both FBG and Fins. The results are shown in Fig. 2E. The HOMA-IR index of mice in the two Ly93 groups was significantly lower than that in the model group ($P < 0.05$).

2.2. Effect of Ly93 on plasma SM and lipid profile in mice

After 12 weeks of treatment, we analyzed the plasma SM levels in mice by an enzyme method reported previously to investigate the effect of Ly93 on SMS activity *in vivo* (Liu et al. 2009). As shown in Fig. 3, Ly93 could decrease the plasma SM concentration in mice in a dose-dependent manner ($P < 0.05$). Mice treated with metformin did nt show any difference to the model group. The plasma HDL-C, LDL-C, TC and TG levels were significantly elevated in the model group compared with the control group ($P < 0.05$). The lipid profile showed no significant difference between the Ly93 groups ($P > 0.05$) and the model group.

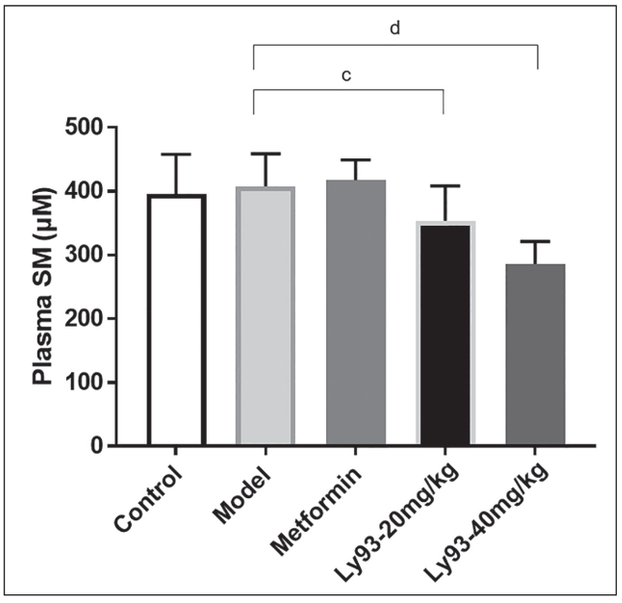

Fig. 3: Effects of Ly93 on plasma SM of C57BL/6 mice. Plasma SM in mice after Ly93 treatment for 12 weeks. Data are means±SD; n = 10 per group; c represents $P < 0.05$ vs model, d represents $P < 0.01$ vs model.

2.3. Effects of Ly93 on oral glucose tolerance test (OGTT) and insulin tolerance test (ITT)

To appraise the glucose and insulin tolerance in mice, the OGTT and ITT were conducted after the treatment. The results were shown in Fig. 4B. Model group demonstrated worse oral glucose tolerance and insulin tolerance compared to control group mice ($P < 0.01$). The insulin tolerance of mice in Ly93-40 mg/kg group was significantly improved ($P < 0.01$), otherwise the oral glucose tolerance was similar with model group (Fig. 4D).

2.4. Effects of Ly93 on liver and skeleton muscle glycogen in mice

The glycogen content was important to evaluate insulin sensitivity of mice, which would be elevated in diabetic animals (Sullivan et

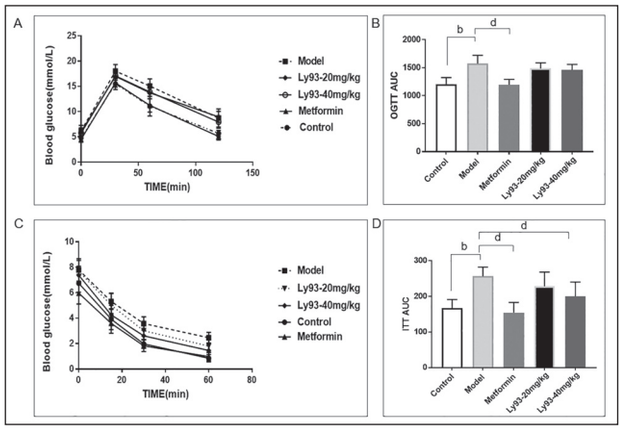

Fig. 4: Effects of Ly93 on oral glucose tolerance and insulin tolerance in C57BL/6 mice. (A) Oral glucose tolerance test (OGTT) was performed on the mice. (B) Results of the OGTT in A are expressed by the area under the curve (AUC). (C) Insulin tolerance test (ITT) was performed on the mice. (D) Results of the ITT in C are expressed by the AUC. Data are means ± SD; n = 10 per group; b represents $P < 0.01$ vs control, d represents $P < 0.01$ vs model.

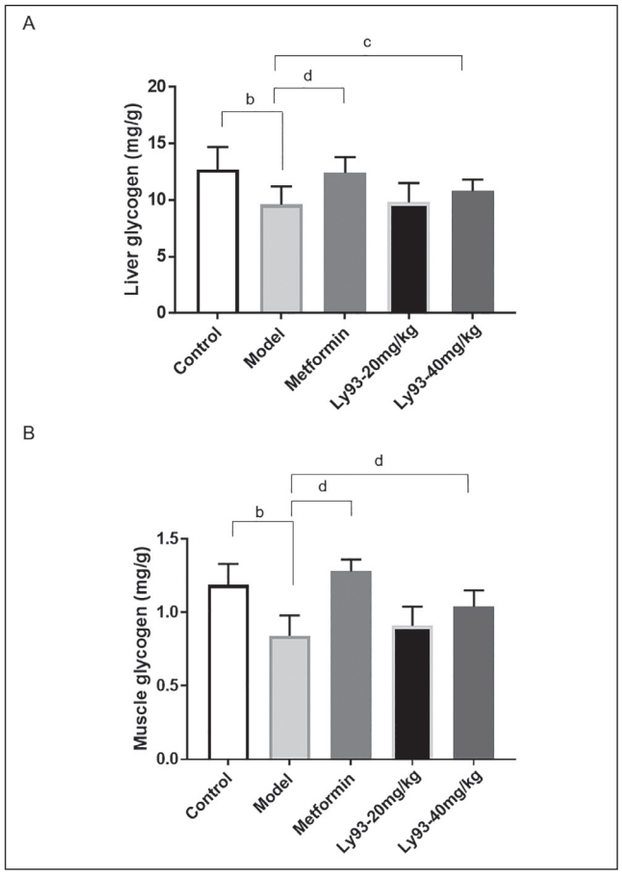

Fig. 5: Effects of Ly93 on liver and muscle glycogen concentration in C57BL/6 mice. After sacrificed, the liver tissues and skeleton muscle tissues of mice were collected immediately for the glycogen concentration determination. (A) Liver glycogen concentration in mice. (B) Muscle glycogen concentration in mice. Data are means±SD; n = 10 per group; b represents $P < 0.01$ vs control, c represents $P < 0.05$ vs model, d represents $P < 0.01$ vs model.

al. 2015). Their liver and skeleton muscle glycogen levels were determined using a commercial kit. As shown in Fig. 5, both the liver and skeleton muscle glycogen levels in model groups were lower than that in the control group ($P < 0.01$). 40 mg/kg Ly93 administration significantly elevated the liver and muscle glycogen content compared with model group ($P < 0.05$), which suggested that Ly93 can regulate insulin sensitivity via glycogen metabolism.

2.5. *Effects of Ly93 on liver insulin signaling in mice*

Insulin signaling regulates glucose metabolism predominantly *via* action on liver, skeletal muscle, and adipose tissue (Boucher et al. 2014). Since the main target organ of Ly93 is the liver, we performed western blot to further investigate the insulin signaling pathway in the liver, by analyzing protein expression level of several key proteins in liver tissue. The phosphorylation of IRS-1, Akt and GSK-3β was determined. The data show that the phosphorylation of IRS-1, Akt and GSK-3β was significantly downregulated in liver tissue in the model group mice compared with the control group (Fig. 6A). However, treatment with Ly93 both 40 mg/kg significantly enhanced the phosphorylation of IRS-1, Akt and GSK-3β.

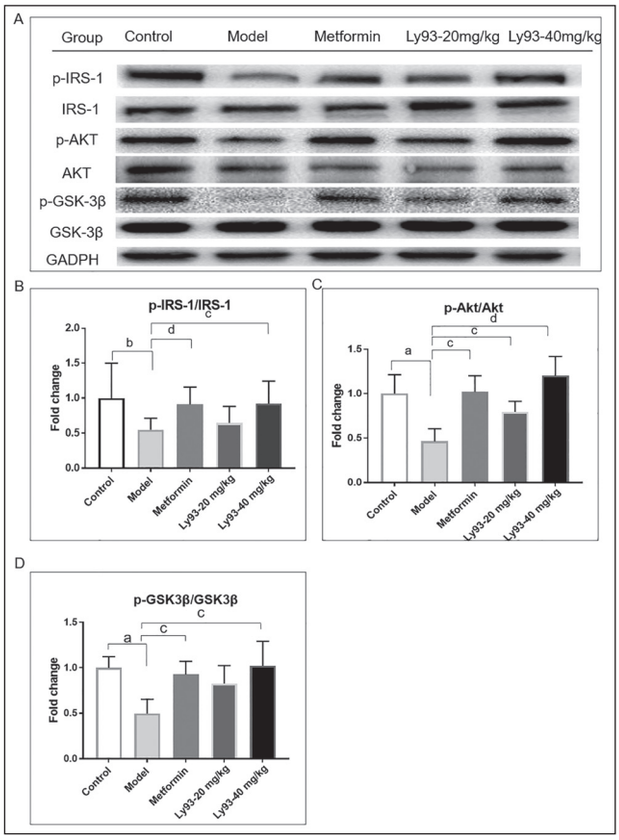

Fig. 6: Effects of Ly93 on insulin signaling in C57BL/6 mice liver. (A) Western blot analysis was performed. Statistically analyses of ratio of (B) phosphorylated IRS-1 and total IRS-1, (C) phosphorylated Akt and total Akt, (D) phosphorylated GSK-3β and total GSK-3β were determined. Data are means ± SD; n = 4-6 per group; a represents $P < 0.05$ vs control, b represents $P < 0.01$ vs control, c represents $P < 0.05$ vs model, d represents $P < 0.01$ vs model.

2.6. *Effect of Ly93 and exogenous SM supplementation on insulin signaling pathway in insulin resistant HepG2 cell*

To better understand the role of SM in the insulin resistance, we added exogenous SM to the medium with or without Ly93. To exploit the effects of Ly93 in HepG2 cells, we initially examined its cytotoxic effects *via* MTT assay. As shown in Fig. 7A, after 48 h treatment , Ly93 less than 12.5 μM was not toxic to HepG2 cells. In consequence, a Ly93 concentration of 12.5 μM was used in subsequent experiments. HepG2 cells were exposed to 10^{-6} M insulin for 24 h to become insulin resistant. Western blot analysis was conducted after Ly93 and exogenous SM treatment. As Fig. 7C shows, Ly93 treatment significantly enhanced the phosphorylation of Akt ($P < 0.05$); this upregulation was significantly reduced by 20 μM exogenous SM ($P < 0.05$).

3. Discussion

There are more than 415 million people living with diagnosed or non-diagnosed diabetes worldwide, among which 90 % are

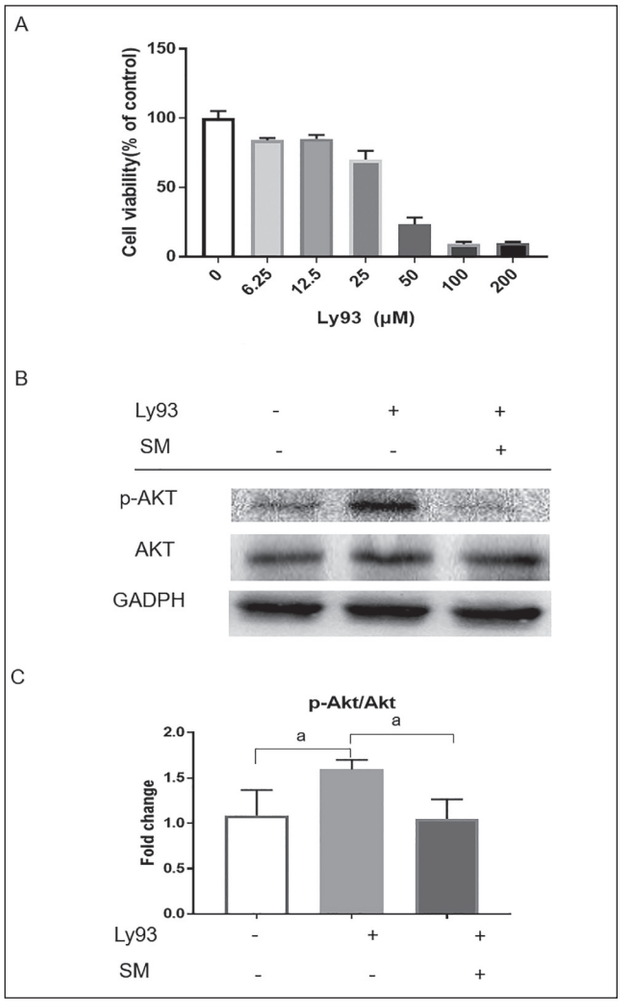

Fig. 7: Effects of Ly93 and exogenous SM supplementation on insulin signaling in HepG2 cells. (A) Cell viability was detected by MTT method after treating with 0 μM, 6.25 μM, 12.5 μM, 50 μM, 100 μM and 200 μM of Ly93 for 48h. (B) Western blot analysis was performed after treating with 12.5 μM of Ly93 with or without 20 μM of SM. (C) Statistically analyses of ratio of phosphorylated Akt and total Akt was determined. Data are means±SD; n = 3 per group; a represents $P < 0.05$

diagnosed with T2D (Chatterjee et al. 2017). Insulin resistance is the major contributor to T2D, with the characteristics of high-level Fins and reduced biological response to insulin (Matulewicz et al. 2016; Patel et al. 2016). The sphingolipid metabolism was considered to be related to diabetes and insulin resistance (Ng et al. 2017). The positive effect of genetic deficiency of SMS2 on insulin resistance has been revealed previously (Li et al. 2011; Sugimoto et al. 2016), but how a small molecule SMS2 inhibitor influences insulin resistance and the insulin signal pathway was not reported before. The main aim of this study was to investigate the role of an SMS2 inhibitor in the insulin resistance developing progress.

After treating with Ly93 for 12 weeks, it was observed that the plasma SM was reduced significantly in both the Ly93-20 mg/kg group and the Ly93-40 mg/kg group (13 % and 30 %, respectively). It was indicated that Ly93 successfully inhibited SMS2 activity in C57BL/6 mice.

In our study, C57BL/6 mice were fed with HFD or ND for 16 weeks to become insulin resistant, and the drug was given *via* intragastric administration during the last 12 weeks at the same time. The model group mice showed abnormally high levels of FBG and Fins, with low levels of glycogen content, and impaired glucose and insulin tolerance was diagnosed, indicating that the model of insulin resistance was induced successfully. We observed a mildly, but not significantly decreased body weight in the Ly93 group. However, Ly93 administration did attenuate the Fins and HOMA-IR index in HFD fed mice. Moreover, the insulin tolerance was improved by Ly93, whereas the effect of Ly93 on the oral glucose tolerance was not so strong, which indicates that the Ly93 treated mice can present better insulin sensitivity with sufficient amounts of insulin. Furthermore, Ly93 increased glycogen content both in liver and muscle. These results suggested that Ly93 administration could partly reverse the insulin resistance induced by HFD.

GSK-3β is a regulator of the activity of glycogen synthase (GS), an enzyme that mediates the conversion of glucose to glycogen. GS is activated due to the phosphorylation of GSK-3β, *via* the action of insulin (Lopez-Soldado et al. 2016; Pandey et al. 2016). We observed that the glycogen content of mice liver and skeleton muscle was decreased in the Ly93 group. It has been reported previously that HFD-fed SMS2 gene knock-out mice showed stronger phosphorylation of Akt and IRβ in the insulin-target organs, and were more insulin sensitive than wild type mice (Sugimoto et al. 2016). But it was unclear if SMS2 inhibitora can work in the same way. To further understand the molecular mechanism of the SMS2 inhibitor, we detected the phosphorylation levels of several key proteins in insulin signal pathway. We found that Ly93 administration increased IRS-1 (Tyr895), Akt and GSK-3β phosphorylation levels in HFD fed mice liver tissue. Our results demonstrated that Ly93 enhanced insulin signaling *via* IRS-1/Akt/GSK-3β in liver and therefore boost the glycogen synthesis.

SM content was reduced by the SMS inhibitor due to the reduction of *de novo* synthesis, and it was reported that SM is potentially a regulator of insulin resistance (Li et al. 2011). However, it is obvious that the SMS inhibitor affects not only the content of SM, but also that of ceramide, phosphatidylcholine (PC) and DAG (Li et al. 2007; Luberto et al. 1998). Ceramide and DAG are both considered to be putative mediators of insulin resistance (Kim et al. 2018; Petersen et al. 2017). In our study, we found that Ly93 increased Akt — the key protein in insulin signal pathway — phosphorylation level in HepG2 cells which was then reversed by exogenous SM. Previous studies had reported that SM plays an important role in cell membrane formation and signal transduction as a platform for signaling molecules (Abe et al. 2014; Li et al. 2007; Perez-Canamas et al. 2017). Our results indicate that Ly93 enhances insulin signaling *via* reducing the SM contents in the cell.

In summary, we are providing results indicating that the SMS2 inhibitor Ly93 improves insulin sensitivity of HFD induced insulin resistant mice by enhancing insulin signaling. As a conclusion, we believe that SMS2 is a potential target for insulin resistance. Before then, it has been shown that the SMS2 inhibitor Ly93 has anti-atherosclerosis activity (Li et al. 2018). Since statin use is considered to be an increased risk of new-onset diabetes, it is possible that SMS2 inhibitors, which have a positive influence on glucose metabolism, could be a potential complement for statin therapy.

4. Experimental

4.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from Hyclone (Logan, UT, USA). Fetal bovine serum (FBS), horse serum, penicillin/streptomycin and trypsin-EDTA were supplied by Gibco (Grand Island, NE, USA). Glucose oxidase activity assay kit was purchased from Aplygen (Beijing, China). SMS2 inhibitor Ly93 (Batch No. CY120928) was given by Professor Ye Deyong from Fudan university. Metformin (Yeason, China).

4.2. Cell culture and animals

HepG2 cells were purchased from the Cell Bank of the Chinese Academy of Sciences, maintained in DMEM supplemented with 10 % FBS at 37 °C, 5 % CO_2 and 1 % penicillin/streptomycin. To evaluate the effect under the insulin resistant condition, HepG2 cells were incubated with 100 mM insulin for 24 h.

Male (C57BL/6) mice aged 8 weeks were purchased from the Animal Center of the School of Pharmacy, Fudan University, Shanghai, China. All animal experiments were approved by the Animal Care and Use Committee of the University of Fudan. Male C57BL/6 mice were randomly divided into 5 groups (n=10), control group were fed with a normal diet (ND), model group were fed with a HFD (40 % fat), metformin group were fed with a HFD and daily treated with 0.2 g/kg metformin, Ly93-20 mg/kg group were fed with a HFD and daily treated with 20 mg/kg Ly93 (10 % DMSO, 30 % PEG-400 and 60 % ddH_2O), Ly93-40 mg/kg group were fed with a HFD and daily treated with 40 mg/kg Ly93 for 12 weeks (i.g).

4.3. Examination of plasma SM and lipid profile in mice

Whole blood samples were collected after the last administration from the orbit and then plasma was separated. The plasma SM in these blood samples were measured according to an enzymatic method reported previously (Liu et al. 2009). The plasma HDL-C, LDL-C, TC and TG levels were measured with commercial kits (Nanjing Jiancheng).

4.4. Determination of FBG, Fins, HOMA-IR, OGTT and ITT in mice

The fasting blood glucose of mice was measured by a Roche glucometer, using blood collected from the mice tails after fasting for 16 h. Fasting blood insulin was measured using an Elisa kit (Mercodia, Sweden). HOMA-IR was determined by the formula: HOMA IR = serum insulin (mmol/L) * (blood glucose (mmol/L)/22.5 (Antunes et al. 2016).

Mice were fasted for 16 h for OGTT and 8 h for ITT. For OGTT, after being injected with 2 g/kg glucose, the mice blood glucose was measured by the glucometer at 0 min, 30 min, 60 min and 120 min. Mice were injected with 0.75 u/kg insulin to conduct ITT, following by blood glucose measured at 0 min, 15 min, 30 min and 60 min.

4.5. Western blot

Treated HepG2 cells were scraped from 6-well were lysed using 100 mL of Pierce RIPA buffer for 30 min. Mice liver tissues were also lysed by RIPA buffer. After centrifuging at 14000 × g for 10 min at 4 °C, we collected the clear supernatant liquid for subsequent measurements. Protein samples were mixed with 6 × loading buffer and boiled at 95 °C for 6 min for protein denaturation. Each sample was loaded into an SDS-polyacrylamide gel, separated by electrophoresis, and then transferred to PVDF membrane. Membranes were blocked in 5 % BSA in TBST for 1 h and then incubated with specific primary antibodies (anti-phospho-Akt Ser473, anti-Akt, anti-phospho-IRS-1 Tyr896, anti-IRS-1, anti-phospho- GSK-3β, anti-GSK-3β and anti-GAPDH, overnight at 4 °C. On the following day, membranes were washed three times by TBST and subsequently incubated with appropriate secondary antibody for 1 h at room temperature. The specific protein bands were visualized using the Amersham Biosciences ECL prime Western blotting detection reagent and ChemiDoc MP imaging system (Bio-Rad). Densitometry analysis was performed using Image-Lab software. Equal loading of protein was ensured by BCA method.

4.6. Liver and skeleton muscle glycogen determination

Liver tissues and muscle tissues separated from euthanized C57BL/6mice legs were investigated to determine if Ly93 treatment increased glycogen deposition. About 1 h after the last administration of treatment, mice were euthanized. The anthrone method was used in the determination of glycogen in liver and muscle(Chun et al. 1998). The commercial glycogen assay kit purchased from Njjcbio was used for glycogen determination.

4.7. Exogenous sphingomyelin supplementation and Ly93 on HepG2

Cell viability was detected by a methyl-thiazol tetrazolium (MTT) assay. HepG2 cells were inoculated into 96-well plates with 2×10^5 cells per well. After treating with 0 μM, 6.25 μM, 12.5 μM, 50 μM, 100 μM and 200 μM of Ly93 for 48 h, cell viability was detected according to the protocol. Sphingomyelins (Sigma) were dissolved in absolute ethanol. HepG2 cells were incubated with 12.5 μM of Ly93 for 17 h and with or without exogenous 20 μM of sphingomyelin for 5 h at the same time. HepG2 cells were treated with 10 nM insulin for 15 min, then Akt phosphorylation was measured by Western blot.

4.8. Statistical analysis

All of the data are typically expressed as means±standard deviation of the mean (SD) and analyzed using GraphPad Prism 7. Data between two groups were analyzed by the unpaired, two-tailed Student's t test. A p value of less than 0.05 was considered statistically significant.

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Conflict of interests. None declared.

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