Fenofibrate Alleviates Insulin Resistance and Hepatic Steatosis via Modulation of let-7/ SERCA2b Axis

Dan Zhang
Kunming Medical University First Affilliated Hospital
Shan-zhuang Niu
Yunnan University
Yi-cheng Ma
Yunnan University
Bo Zhou
Kunming Medical University
Yi Deng
Kunming Medical University
Zuo-tao Geng
Lijiang Woman's and Children's Hospital
Yu Wen
Kunming Medical University First Affilliated Hospital
Su-feng Luo
Kunming Medical University First Affilliated Hospital
Chun-jing Shi
Kunming Medical University First Affilliated Hospital
Guang-yu Pu
Kunming Medical University First Affilliated Hospital
Cheng-gang Zou
Yunnan University
Yuan-Li Chen (402949806@qq.com)
Department of Faculty of Basic Medicine, Kunming Medical University, Kunming 650500, China
https://orcid.org/0000-0001-9269-2486
Lan-qing Ma
Kunming Medical University First Affilliated Hospital

Research

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Abstract

Background: Fenofibrate is a peroxisome proliferator-activated receptor alpha agonist, which is widely used in clinical practice to effectively ameliorates the development of NAFLD. However, the molecular mechanism remains largely unknown, the present study aimed to investigate the role and specific mechanism of fenofibrate on lipid metabolism disorders associated diseases.

Methods: The male C57BL6/J mice were divided into 3 groups, the mice in control group (*n*=10) were fed with normal chow diet, and the mice in HFD-fed group (*n* =10) were fed with a high fat diet (HFD) for 14 weeks. For the fenofibrate +HFD-fed group (*n* =10), the mice fed HFD were orally gavaged with fenofibrate (40 mg/kg) daily for the last 4 weeks. Body weight and hip width were measured. Macrosteatosis and fat deposition in the liver were measured by H&E staining and Oil red O staining individually. The levels of serum and hepatic triglyceride were measured, and HOMA-IR, HOMA-ISI were analyzed. The levels of SCD-1, Bip, CHOP and SERCA2b were measured by western blotting. The expression of let-7 were analyzed by qPCR, and the complementarity between the 3'-UTR of SERCA2b gene and let-7 was measured by luciferase reporter assay.

Results: Fenofibrate reduces hepatic steatosis and insulin resistance in HFD-fed mice. Fnofibrate alleviates endoplasmic reticulum stress (ER stress) of mice fed a high fat diet (HFD). Fenofibrate increases the levels of Sarco-endoplasmic reticulum Ca^{2+}-ATPase 2b (SERCA2b) which serves as a regulator of ER stress. Further, the levels of let-7 microRNA is also regulated by fenofibrate, and let-7 directly targets 3’-UTR of SERCA2b.

Conclusion: The present data suggests that fenofibrate alleviates ER stress through the let-7/SERCA2b axis to protect against excessive lipid accumulation in the liver of Non-alcoholic fatty liver disease (NAFLD) mice.

Background

Non-alcoholic fatty liver disease (NAFLD) is one of the most common chronic liver diseases worldwide now [1]. NAFLD is typically characterized by excess accumulation of abnormal amounts of fat in the hepatocytes [2]. The excessive fat accumulation in the liver is strongly associated with an increased risk of obesity, type 2 diabetes, metabolic syndrome, cardiovascular disease and cancer [3,4]. NAFLD has become an important public health issue because of its high prevalence and potential progression to severe liver diseases. Therefore, understanding of the mechanism of NAFLD development is driving progress in therapeutic strategies.

Accumulating evidence reveals that hepatic endoplasmic reticulum (ER) stress (ER stress) critically contributes to pathogenesis of NAFLD [5]. ER present in the cytoplasm of eukaryotic cells carries out multiple essential functions, which is particularly important in calcium storage, protein and lipid synthesis [6]. ER stress occurs when ER function is disturbed by misfolded proteins accumulation or ER calcium is depleted. Various pathological conditions, including hypoxia, ischaemia, inflammation, energy

disturbance, and oxidative stress, interrupt the homeostatic function of ER and then trigger the ER stress [6,7]. It is well known that ER stress activates a signal transduction system termed the unfolded protein response (UPR) [8]. Activation of the UPR depends on three ER stress sensor proteins: inositol-requiring enzyme 1α (IRE1α), protein kinase RNA-like ER kinase (PERK), and ATF6, a transmembrane basic leucine zipper transcription factor [9,10]. For instance, activated IRE1α specifically cleaves the mRNA of the transcription factor X-box binding protein (XBP)1, which is required for translation of transcriptionally active XBP1[10]. During ER stress, the release of ATF6 from the ER chaperon Grp78/BiP permits activation of ATF6, mediating the expression of UPR target genes, including XBP-1. Although the role of ER stress in NAFLD was confirmed [11], the specific mechanism remains largely unknown. Sarco-endoplasmic reticulum calcium ion ATPase (SERCA) is a P-type ATPase family member that pumps Ca^{2+} from the cytosol to the ER [12]. Among family numbers of SERCAs, SERCA2b serves as a housekeeping isoform involved in the cellular calcium homeostasis [13]. The SERCA2b protein levels and activity are significantly reduced in mice with obesity [14,15]. Overexpression of SERCA2b ameliorates ER stress, increases glucose tolerance and improves the NAFLD phenotypes in obese mice [14-16]. These results indicate that SERCA2b plays an important role in regulating ER stress during NAFLD.

MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the post-transcriptional level by targeting sequence motifs located within the 3' untranslated region (UTR) of mRNA transcripts [17]. A number of studies have shown that disturbance of miRNA expressions contributes to metabolic disorders associated with NAFLD by altering key signaling elements [18-22]. For instance, while overexpression of the let-7 in mice resulted in impaired glucose tolerance, knockdown of the let-7 in mice resulted in hepatic lipid accumulation and improved insulin resistance in mice fed HFD [23]. The action of let-7 is mainly due to inhibition of the Insulin-PI3K pathway by targeting insulin receptor (INSR) and insulin receptor substrate 2 (IRS2) and insulin-like growth factor 1 receptor (IGF1R) [23,24]. Our recent study demonstrates that miR-30b induces ER stress by targeting SERCA2b, thereby impairing insulin sensitivity in rats fed HFD [20]. Thus, miRNAs represent potential novel therapeutic targets for NAFLD. Fenofibrate, the peroxisome proliferator-activated receptors α (PPAR-α) agonist, is a prescription medication used to lower cholesterol and triglycerides [25]. It has been shown that fenofibrate improves fibrosis, inflammation and lipid homeostasis of hepatic by activing PPAR-α [26], but the precise mechanism is not well understood. In this study, we showed that fenofibrate treatment suppressed hepatic lipid accumulation and improved insulin resistance by suppressing let-7 expression in the liver of mice fed HFD. Our results identified that let-7 family miRNAs were regulators of SERCA2b by targeting 3’-UTR of SERCA2b mRNA. Thus, fenofibrate alleviates ER stress through the let-7/SERCA2b axis to protect against hepatic lipogenesis of NAFLD mice.

Materials And Methods

Animals

Adult (age, 6 weeks) male C57BL6/J mice were obtained from the Nanjing Biomedical Research Institute of Nanjing University (Nanjing, China). The mice were fed with free access to water and food in plastic

cages at a controlled temperature of 20± 2℃, humidity of 50%–60% and a 12-hour light–dark cycle. After 1 week of acclimatization, the mice were randomly divided into 3 groups: control group (*n*=10), HFD-fed group (*n*=10), and fenofibrate + HFD-fed group (*n*=10), fenofibrate was dissolved with 0.5% sodium carboxymethyl cellulose (CMC-Na). The mice in control group were fed with normal chow diet, and the mice in HFD-fed group were fed with a high fat diet (HFD), which consists of 20% carbohydrate, 20% protein and 60% fat (total 25.07 kJ/g), for 14 weeks. For the fenofibrate +HFD-fed group, the mice fed HFD were orally gavaged with fenofibrate (40 mg/kg) daily for the last 4 weeks. Body weight was measured once a week throughout the investigation.

Western blotting

Mouse liver was homogenized in liquid nitrogen, the homogenate was lysed on ice for 1 h in lysis buffer (BioTeKe, Beijing, China). Protein lysates were loaded into each well and separated on 7.5%, 10% or 12.5% SDS polyacrylamide gel. Separated proteins were then transferred to immobilon-PSQ transfer PVDF membrane (Millipore, Bedford, MA), and blocked with 5% skim milk in Tris-buffered saline supplemented with 0.1% Tween-20 (TBST) for 2 hours at room temperature. The primary antibodies were anti-ATP2A2/SERCA2b (#4388, Cell Signaling Technology, Beverly, MA), anti-Bip (#3183, Cell Signaling Technology), anti-CHOP (#2895, Cell Signaling Technology), and anti-GAPDH antibodies (#8884, Cell Signaling Technology). The immunoreactive signals were detected using the ECL western blotting substrate reagents (#32109, Thermo Scientific Science, Waltham, MA). An imaging system (Amersham Imager 600) was used for documentation of the western blotting results. Quantitation was analyzed with the ImageJ (NIH).

Quantitative RT-PCR

Total miRNAs in tissue were extracted with the miRcute miRNA Isolation kit (DP501, Transgen, Being, China). cDNA was reversely synthesized using the miRcute Plus miRNA First-Strand cDNA kit (KR211-01, Trangen, Being, China). Quantitative real-time PCR (qRT-PCR) analysis of let-7a, let-7b, let-c, let-7d, let-7e, let-7f, let-7g, let-7i, and mir-98 were performed using miRcute Plus miRNA qPCR kit (SYBR Green) (FP411-01-01,Trangen, Being, China) on a Roche LightCycler 480 System (Roche Applied Science, Mannheim, Germany). The primers used for PCR were as follows:

let-7a: 5’- UGAGGUAGUAGGUUGUAUAGUU- 3’;

let-7b: 5’- UGAGGUAGUAGGUUGUGUGGUU- 3’;

let-7c: 5’- UGAGGUAGUAGGUUGUAUGGUU - 3’;

let-7d: 5’- AGAGGUAGUAGGUUGCAUAGUU- 3’;

let-7e: 5’- UGAGGUAGGAGGUUGUAUAGUU- 3’;

let-7f: 5’- UGAGGUAGUAGAUUGUAUAGUU- 3’;

let-7g: 5’- UGAGGUAGUAGUUUGUACAGUU- 3’;

let-7i: 5’- UGAGGUAGUAGUUUGUGCUGUU- 3’;

mir-98: 5’- UGAGGUAGUAAGUUGUAUUGUU- 3’.

All qRT-PCRs were performed in triplicates. U6 was used for normalization.

Luciferase reporter assay

The 3'-UTR of SERCA2b mRNA was amplified by PCR and cloned into the psiCHECK2 luciferase reporter vector. The human hepatocarcinoma cell line HepG2 cells were grown in 24-well plate containing Dulbecco’s Modified Eagle Medium (Hyclone, Logan, UT) supplemented with 10% Fetal Bovine Serum (FBS, Hyclone), and maintained at 37°C with 5% CO2. When 60% to 80% confluent, the cells were transfected using Lipofectamine 2000® Reagent (Thermo Fisher Scientific, Waltham, MA) with 3'-UTR of SERCA2b reporter plasmids with negative control (NC) mimics or miR-let-7 mimic. At 48 h transfection, the luciferase activity was determined by the Dual Luciferase Assay System (Promega, Beijing, China). The renilla luciferase activity was used as a normalization in each well. All experiments were repeated three times.

Hematoxylin and eosin (H&E) staining

Fresh liver samples were fixed in 10% formalin and embedded in paraffin. After sectioned at a 5-μm thickness, and the sections were mounted onto glass microscope slides, and air-dried at room temperature for 24 h. The sections were then stained with hematoxylin and eosin (H&E). Images were acquired in a Leica aperio CS2 system.

Oil-red O staining

Oil-red O staining was used to determine lipid deposition. In briefly, OCT-embedded tissues were sectioned at a 10-μm thickness, and fixed in 10% formalin for 10-15 min, then rinsed in distilled water and air-dried. The sections were stained with freshly prepared Oil Red O staining solution (Sigma-Aldrich) for 8–10 min at 60℃. Finally, the sections were counterstained with hematoxylin. Images were acquired in a Leica aperio CS2 system.

Statistics

The data presented in each figure are mean ± SD of three independent experiments performed in triplicate. The data presented in each figure are mean ± SD. Statistical differences between two groups were analyzed using Student’s t-test. Statistical difference between multiple groups were performed by one-way ANOVA, followed by a Student-Newman–Keuls test. Data were analyzed using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA), A value of $P < 0.05$ was considered to represent a statistical significance.

Results

Fenofibrate reduces hepatic steatosis and insulin resistance in HFD-fed mice

Mice became obviously obese, which were accompanied with significant increases in body weight and hip width after the 14 weeks of HFD feeding (Fig. 1a-b). To confirm the beneficial effects of fenofibrate, these HFD-induced obese (DIO) mice were administered vehicle (CMC-Na) and fenofibrate by oral gavage daily for 4 weeks. We found that the body mass and the average hip width of DIO mice were significantly decreased after fenofibrate administration. Compared with the vehicle, DIO mice treated with fenofibrate exhibited significant decreases in hepatocyte ballooning (Fig. 1c), macrosteatosis (H&E staining) and fat deposition (Oil red O staining) in the liver (Fig. 1d) as well as the levels of serum and hepatic triglyceride (TG) (Fig. 1f-g). Next, we measured the protein levels of steroyl-CoA desaturase (SCD-1), which is a key enzyme for the synthesis of monounsaturated fatty acids [27]. The protein levels of SCD-1 were significantly up-regulated in the liver of DIO mice, which were reduced by fenofibrate treatment (Fig. 1e). As insulin resistance (IR) is one of the most frequent complications of obesity, we determined the effects of fenofibrate on IR. We found that fenofibrate treatment reduced HOMA-IR (Homeostatic model assessment for insulin resistance) (Fig. 1h), and alleviated HOMA-ISI (Homeostatic model assessment for insulin sensitivity) (Fig. 1i). These data indicate that fenofibrate treatment dramatically improves abnormal lipid accumulation and insulin resistance in the liver of DIO mice.

Fenofibrate treatment ameliorates ER stress accompanied by up-regulation of SERCA2b in the liver of HFD-fed mice

ER stress plays a vital role in the pathological development of hepatic steatosis [5]. We thus tested whether ER stress activated by HFD feeding were attenuated by fenofibrate treatment. We determined the protein levels of Bip and CHOP, two indicators of ER stress by western blotting, and found that both protein expressions were up-regulated in the in the liver of DIO mice (Fig. 2a-b), compared with those in mice fed normal diet. Fenofibrate treatment dramatically ameliorates ER stress, which was activated by HFD feeding (Fig. 2a-b). It has been shown that the protein expression or activity of SERCA2b are significantly reduced in mice with obesity [14,15,20]. whereas increased SERCA2b expression or activity contributes to the amelioration of ER stress in obese mice [28,29]. To identify whether fenofibrate ameliorates ER stress by regulating SERCA2b, we measured the protein levels of SERCA2b by western blotting. Indeed, fenofibrate treatment significantly restored the protein expression of SERCA2b in the liver of DIO mice (Fig. 2c). Similar results were confirmed by immunohistochemistry analysis (Fig. 2d). These data indicate that fenofibrate treatment ameliorates ER stress, at least in part, by up-regulating SERCA2b in the liver of HFD-fed mice.

Fenofibrate treatment attenuates let-7 expression in the liver of HFD-fed mice

Overexpression of let-7 in mice results in impaired glucose tolerance, while global knockdown of let-7 improves the impaired glucose tolerance in mice with obesity [30]. Using miRNA microarray, our recent study reveals that the levels of let-7 family miRNAs are elevated in the liver of rats fed HFD [20]. We

measured the levels of let-7 family in the liver of mice by RT- qPCR, and found that the levels of let-7a, let-7b, let-c, let-7d, let-7e, let-7f, let-7g, and let-7i were increased in DIO mice (Fig. 3a). Fenofibrate treatment significantly inhibited the levels of let-7 family in the liver of DIO mice.

SERCA2b is a target gene of let-7

let-7 is highly conserved in different species including human and rodents (Fig. 3b). To identify whether SERCA2b is regulated by let-7, we first analyzed the sequence alignment of SERCA2b 3'-UTR and let-7 family by Targetscan algorithm. We identified let-7 family that could bind to the 3'UTR of SERCA2b (Fig. 3c). To confirm the involvement of let-7 in the regulation of SERCA2b expression, we construct a luciferase reporter vector containing the 3'-UTR of SERCA2b. We found that transfection of let-7 mimics markedly decreased the luciferase activity of SERCA2b 3'-UTR in HepG2 cells (Fig. 3d). The data clearly demonstrates that let-7 miRNA directly targets SERCA2b.

Discussion

In this study, we characterized the molecular mechanism underlying fenofibrate alleviated hepatic steatosis and insulin resistance in a rodent model of NAFLD (Fig. 4). Fenofibrate reduces the expressions of let-7 family, which are up-regulated in the liver of DIO mice. Our results identify that SERCA2b is a target of let-7. Downregulation of SERCA2b *results in the activation of* ER stress, thereby inducing steatosis and insulin resistance. Thus, fenofibrate improves the pathogenesis of NAFLD by modulation of the let-7/ SERCA2b axis.

Activation of ER stress initiates inflammation, insulin resistance, and hepatic steatosis, all of which are important factors involved in NAFLD pathogenesis. Fenofibrate can alleviate endoplasmic reticulum stress in the liver of mice with obesity [31], but the mechanism remained largely unclear. In this study, our results demonstrate that fenofibrate can alleviate ER stress via up-regulation of SERCA2b. It has been shown that overexpression of SERCA2b significantly suppresses steatosis, at least in part, by inhibiting the up-regulation of lipogenesis genes, such as SCD1, DGAT2, and ACC2 in the liver of ob/ob mice [14]. However, overexpression of SERCA2b does not influence expression of SREBP1c, a master regulator of these lipogenic genes (SCD1, DGAT2, and ACC2). Thus, downregulation of these lipogenic genes by SERCA2b is independent of SREBP1c. Actually, XBP1 directly regulates the expression of lipogenic genes, such as SCD1, DGAT2, and ACC2 in the liver [32]. Thus, SERCA2b regulates lipid accumulation probably by inhibiting lipogenic genes mediated by XBP1.

Dysregulation of miRNA expressions has been associated with NAFLD by altering a variety of pathways [18-20,22]. For example, using miRNA microarray, our recent study identifies that upregulation of miR-30b promotes insulin resistance and hepatic steatosis by targeting SERCA2b in rats fed HFD [20]. Interestingly, elevated expressions of let-7 family are observed in the liver of the rats fed HFD [20]. In the current study, the levels of let-7 family, such as let-7a, let-7b, let-c, let-7d, let-7e, let-7f, let-7g, let-7i, and mir-98, were also observed in the liver of DIO mice. Meanwhile, we found that fenofibrate treatment downregulated the expressions of let-7 family in the DIO mice. Two previous studies have demonstrated

that let-7 induces hepatic lipid accumulation and insulin resistance by targeting the key components of the insulin signaling pathway, such as INSR, IRS2, and IGF1R in mice with obesity [23]. Our data demonstrated that SERCA2b is a novel target of let-7. Thus, our results revealed that fenofibrate improves the pathogenesis of NAFLD by alleviating ER stress through let-7/ SERCA2b axis.

Strength And Limitations

The strengths of this study were listed as following: First, we clarify that fenofibrate alleviates ER stress via up-regulation of SERCA2b, thereby improving steatosis and insulin resistance in a rodent model of NAFLD. Second, the data also suggests that fenofibrate reduces the expressions of let-7 family, which are up-regulated in the liver of NAFLD mice, and further research identify that SERCA2b is a target of let-7. Thus, Fenofibrate alleviates insulin resistance and hepatic steatosis via modulation of let-7/ SERCA2b axis. This study also has some limitations. Clearly, the mechanism underlying fenofibrate downregulates let-7 need to be investigated further in light of our current results.

Conclusions

In conclusion, our data demonstrates that fenofibrate alleviates ER stress by regulating SERCA2b in the liver of NAFLD mice. Moreover, the levels of let-7 is also regulated by fenofibrate, and let-7 regulates SERCA2b by directly targeting the 3'-UTR of SERCA2b. Thus, fenofibrate improves the pathogenesis of NAFLD by modulation of the let-7/ SERCA2b axis.

Abbreviations

ER stress: endoplasmic reticulum stress; HFD: high fat diet; SERCA2b: Sarco-endoplasmic reticulum Ca^{2+}-ATPase 2b; NAFLD: Non-alcoholic fatty liver disease; UPR: unfolded protein response; IRE1α: inositol-requiring enzyme 1α; PERK: protein kinase RNA-like ER kinase; XBP: X-box binding protein; INSR: insulin receptor; IRS2: insulin receptor substrate 2; IGF1R: insulin-like growth factor 1 receptor; PPAR-α: peroxisome proliferator-activated receptors α; CMC-Na: sodium carboxymethyl cellulose; DIO: HFD-induced obese; TG: triglycerides; IR: insulin resistance; HOMA-IR: Homeostatic model assessment for insulin resistance; HOMA-ISI: Homeostatic model assessment for insulin sensitivity

Declarations

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Authors' contributions

All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. ZD, NSZ, MLQ, CYL and ZCG involved in study concept and design. ZD and ZB performed the animal experiment and acquired the data. NSZ and MYC performed the cell experiment and acquired the data. DY and GZT helped with the animal experiments. WY and LSF interpreted the results and drew the figures. CYL, SCJ and PGY were involved in drafting of manuscript, study supervision, revision of manuscript for intellectual content. All authors read and approved the final manuscript.

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Availability of data and materials

The dataset used and analyzed during the current study is available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Ethical approval for this animals' research was given by the Ethics Committee of the Kunming Medical University (No: kmmu2020248).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]The First Affiliated Hospital, Yunnan Institute of Digestive Disease, Kunming Medical University, Kunming 650032, China. [2]State Key Laboratory for Conservation and Utilization of Bio-Resources in Yunnan, School of Life Sciences, Yunnan University, Kunming 650091, China. [3]Department of Faculty of Basic Medicine, Kunming Medical University, Kunming 650500, China. [4]Department of Surgery, Lijiang Women's and Children's Hospital, Lijiang 530721, China.

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Figures

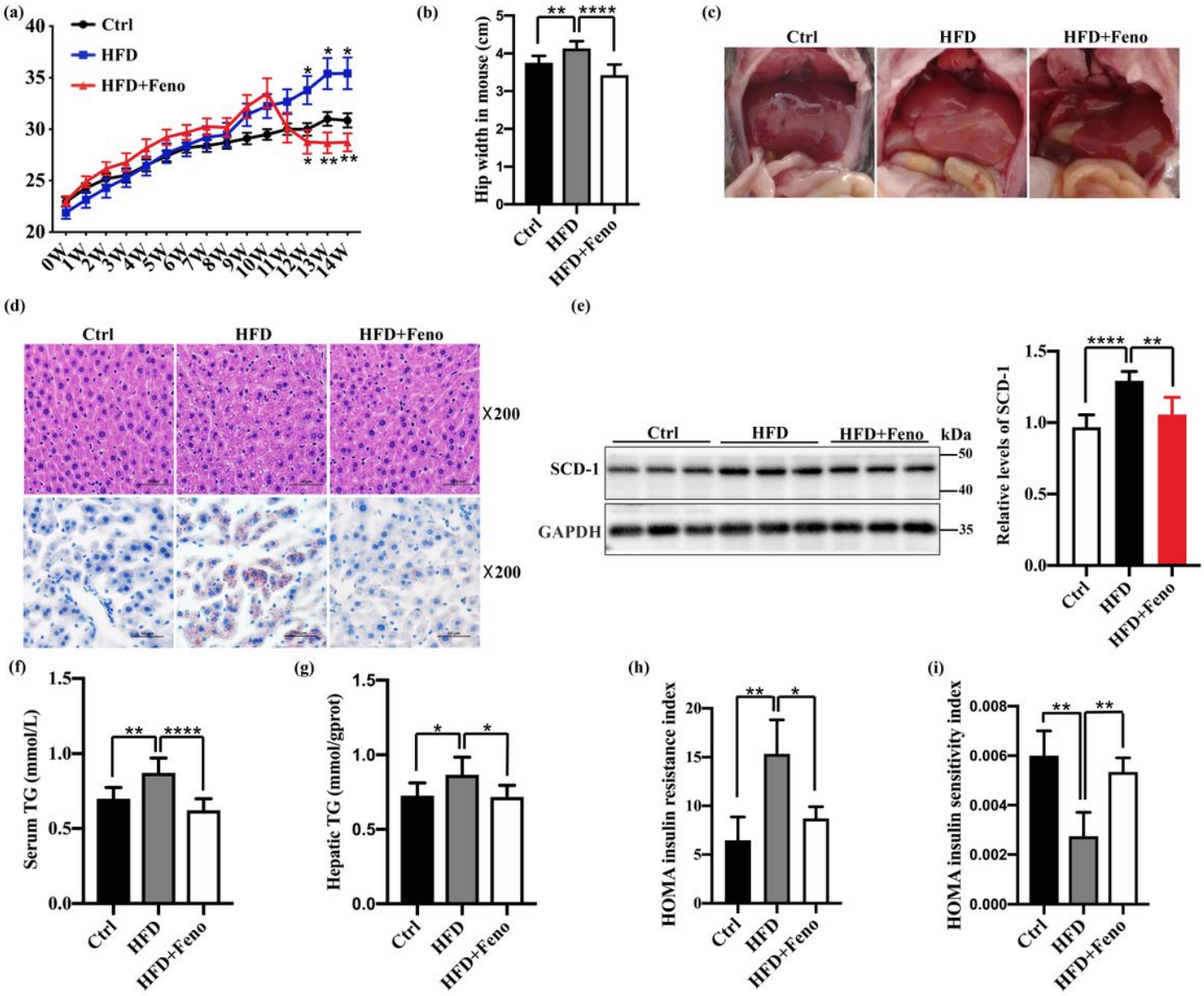

Figure 1

Effects of fenofibrate on body mass, liver histology, lipid accumulation and IR in HFD-fed male C57/BL6 mice. Effects of fenofibrate on body mass (a), hip width (b). and liver gross morphology (c) of mice fed HFD. These results are means ± SD of 10 mice in each group. *P < 0.05, **P < 0.01, ****P < 0.0001, HFD group versus control group, or fenofibrate +HFD-fed group versus HFD group. (d) Pathological changes in

the liver were evaluated with H&E and Oil-red O staining (×200) respectively. Representative images were shown. (e) The levels of SCD-1 were measured by western blotting. Representative Western blots are shown. These results are means ± SD of 10 mice in each group. **P < 0.01, ****P < 0.0001, HFD group versus control group, or fenofibrate+HFD-fed group versus HFD group. (f) Serum triglyceride (TG) levels. These results are means ± SD of 10 mice in each group. **P < 0.01, ****P < 0.0001, HFD group versus control group, or fenofibrate +HFD-fed group versus HFD group. (g) Hepatic triglyceride (TG) levels. These results are means ± SD of 10 mice in each group. *P < 0.05, HFD group versus control group, or fenofibrate +HFD-fed group versus HFD group. (h) HOMA insulin resistance index. These results are means ± SD of 10 mice in each group. *P < 0.05, **P < 0.01, HFD group versus control group, or fenofibrate +HFD-fed group versus HFD group. (i) HOMA insulin sensitivity index. These results are means ± SD of 10 mice in each group. **P < 0.01, HFD group versus control group, or fenofibrate +HFD-fed group versus HFD group. P-values were calculated using a one-way ANOVA followed by a Student-Newman-Keuls test.

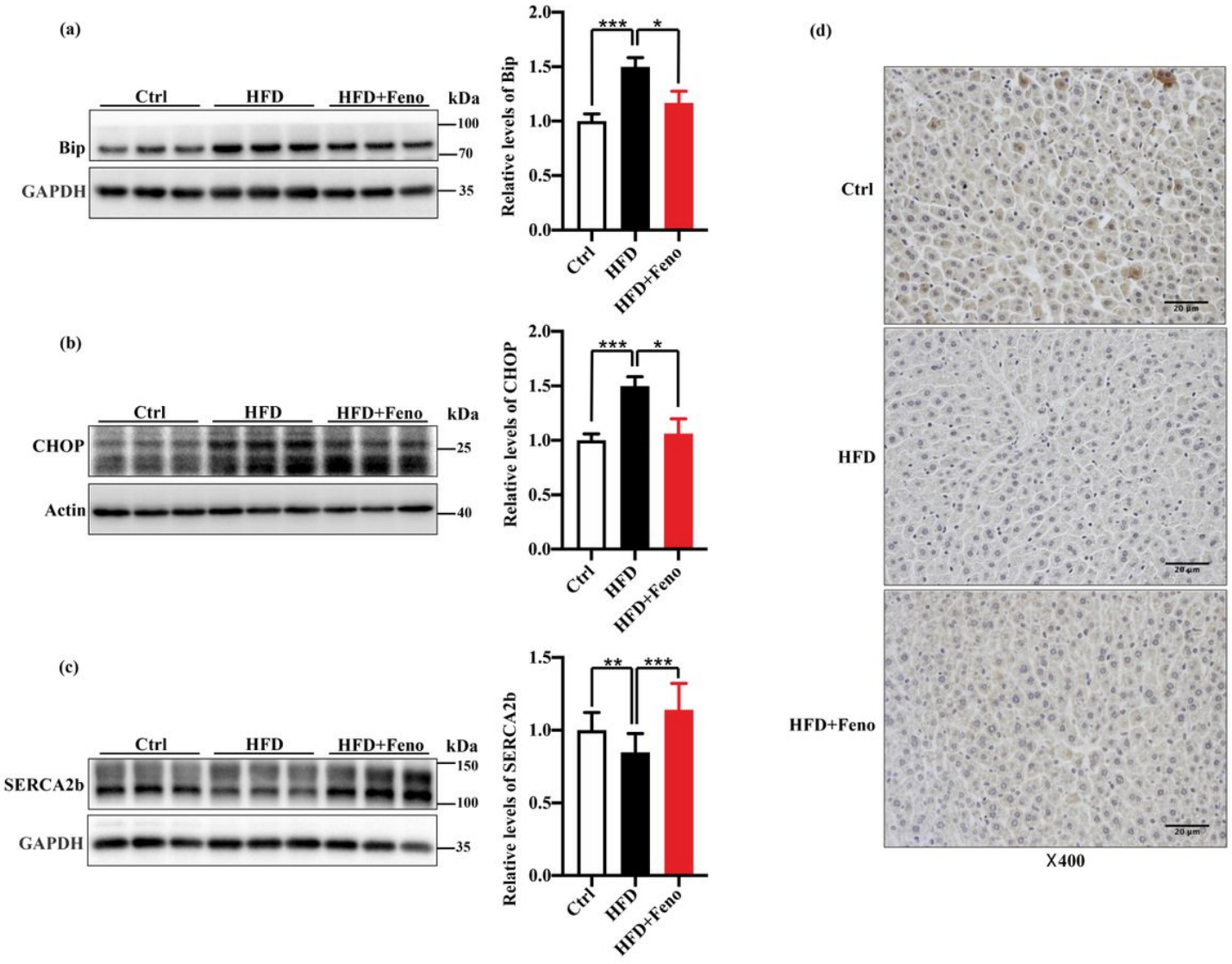

Figure 2

Effects of fenofibrate on ER stress and the expression of SERCA2b in the liver of HFD-fed male C57/BL6 mice. The levels of Bip (a), CHOP (b), and SERCA2b (c) were measured by western blotting. Representative Western blots are shown. These results are means ± SD of 10 mice in each group. *P < 0.05, **P < 0.01, ***P < 0.001, HFD group versus control group, or fenofibrate +HFD-fed group versus HFD group. (d)The level of SERCA2b in the liver was determined by immunohistochemistry (×400).

Representative images were shown. P-values were calculated using a one-way ANOVA followed by a Student-Newman-Keuls test.

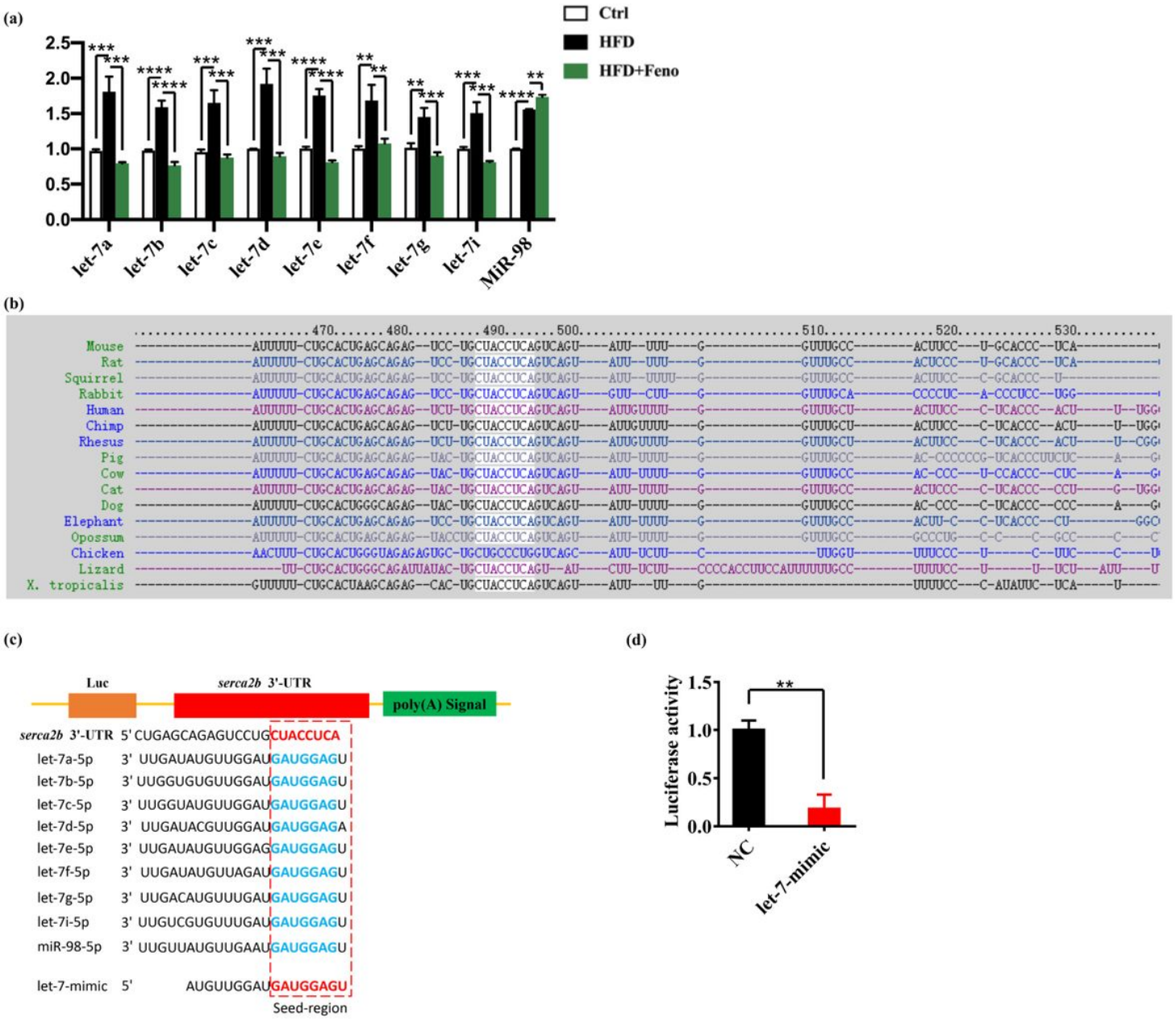

Figure 3

Let-7 regulates SERCA2b in the liver of HFD-fed male C57/BL6 mice. (a) qPCR analysis confirmed that fenofibrate attenuated let-7 expression in the liver of HFD-fed mice. These results are means ± SD of 10 mice in each group. **P < 0.01, ***P < 0.001, ****P < 0.0001, HFD group versus control group, or fenofibrate +HFD-fed group versus HFD group. (b) Sequences of the let-7 family in different species. (c)

Complementarity between the 3'-UTR of SERCA2b gene and let-7. The box indicates the seed region. (d) Luciferase analysis of a reporter vector harbouring the 3'-UTR of SERCA2b in HepG2 cells transfected with negative control (NC) or let-7 mimics for 48 h. These results are means ± SD of three independent experiments. **P < 0.01, let-7 mimics group versus negative control group. P-values were calculated using a one-way ANOVA followed by a Student-Newman-Keuls test.

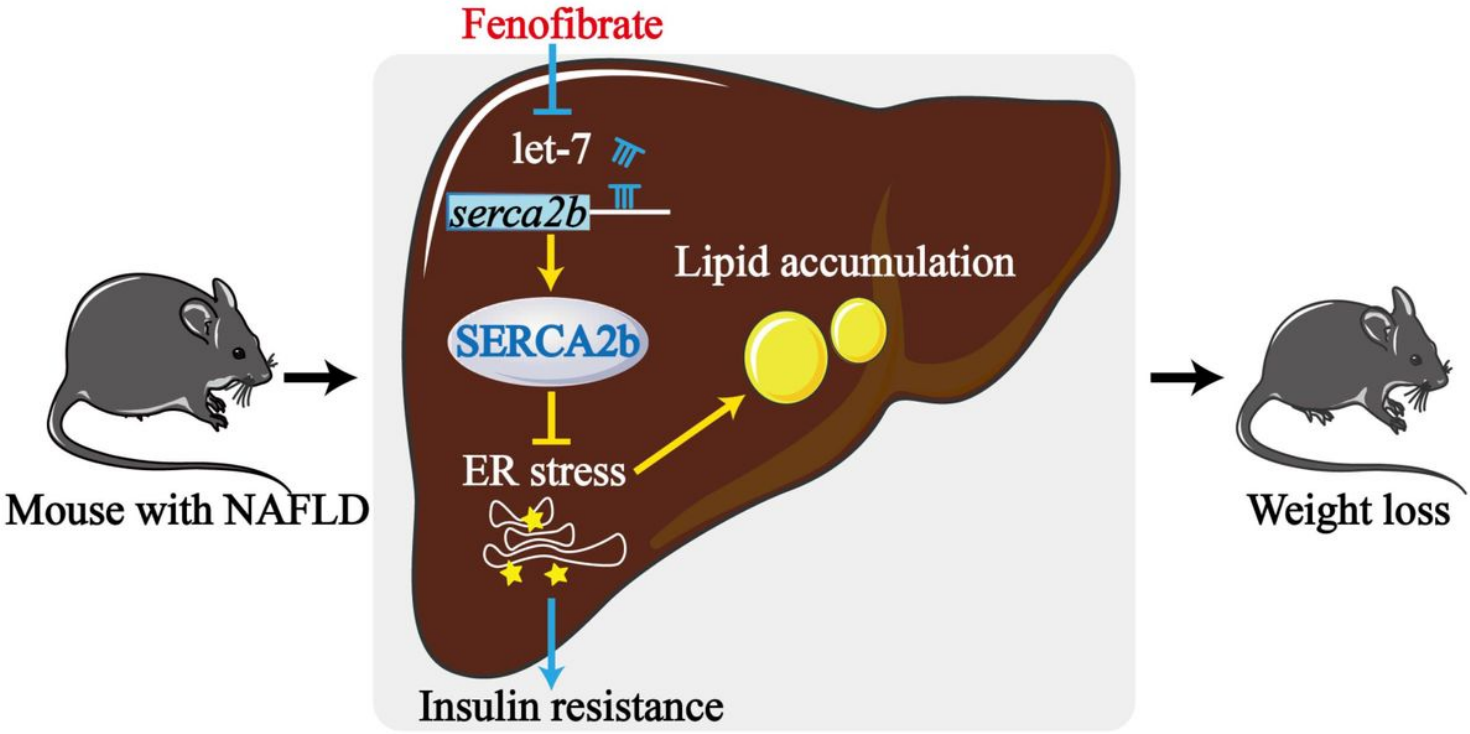

Figure 4

Proposed mechanism by which fenofibrate improves insulin resistance and hepatic lipid accumulation