

Erythropoietin suppresses hepatic steatosis and obesity by inhibiting endoplasmic reticulum stress and upregulating fibroblast growth factor 21

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Abstract. Erythropoietin (EPO), known primarily for its role in erythropoiesis, was recently reported to play a beneficial role in regulating lipid metabolism; however, the underlying mechanism through which EPO decreases hepatic lipid accumulation requires further investigation. Endoplasmic reticulum (ER) stress may contribute to the progression of hepatic steatosis. The present study investigated the effects of EPO on regulating ER stress in fatty liver. It was demonstrated that EPO inhibited hepatic ER stress and steatosis *in vivo* and *in vitro*. Interestingly, these beneficial effects were abrogated in liver-specific sirtuin 1 (SIRT1)-knockout mice compared with wild-type littermates. In addition, in palmitate-treated

hepatocytes, small interfering RNA-mediated SIRT1 silencing suppressed the effects of EPO on lipid-induced ER stress. Additionally, EPO stimulated hepatic fibroblast growth factor 21 (FGF21) expression and secretion in a SIRT1-dependent manner in mice. Furthermore, the sensitivity of hepatocytes from obese mice to FGF21 was restored following treatment with EPO. Collectively, the results of the present study revealed a new mechanism underlying the regulation of hepatic ER stress and FGF21 expression induced by EPO; thus, EPO may be considered as a potential therapeutic agent for the treatment of fatty liver and obesity.

Introduction

Non-alcoholic fatty liver disease (NAFLD) has become a major public health concern, affecting over half a billion people worldwide, and has been associated with a variety of metabolic comorbidities (1). Hepatic steatosis, as a key metabolic hallmark of NAFLD, is mainly caused by excessive fat accumulation. An overload of free fatty acids, particularly saturated free fatty acids, may induce the endoplasmic reticulum (ER) stress response (2,3). Additionally, the presence of non-functional unfolded or misfolded proteins and subsequent ER stress may result in reduced lipid droplet stability and increased lipogenesis, aggravating NAFLD (4). Importantly, in the livers of patients with NAFLD and animal models of diet-induced obesity, chronic ER stress and prolonged activation of the unfolded protein response (UPR) was observed; these factors play a key role in the development of hepatic steatosis (5-7).

Erythropoietin (EPO), a glycoprotein hormone, has been traditionally considered as a key regulator of erythropoiesis (8,9); however, its protective role has recently been extended to ameliorating metabolic disorders (10). Specifically, EPO was demonstrated to promote brown fat-like characteristics to increase fatty acid oxidation in white adipose tissue from obese mice (11); EPO was reported to decrease adipose tissue mass by increasing fat utilization in skeletal muscles from obese mice or humans during aerobic exercise (12,13). In liver tissues, EPO was found to reduce insulin resistance via peroxisome proliferator-activated receptor γ (PPAR γ)-dependent protein kinase B (AKT) activation, and alleviate steatosis, partially via

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Abbreviations: EPO, erythropoietin; ER, endoplasmic reticulum; eIF2 α , eukaryotic initiation factor 2 α ; FGF21, fibroblast growth factor 21; HFD, high-fat diet; IPGTT, intraperitoneal glucose tolerance test; NAFLD, non-alcoholic fatty liver disease; PA, palmitate; PDI, protein disulfide isomerase; GRP78, glucose-regulated protein 78; PERK, protein kinase-like ER kinase; PGC-1 α , peroxisome proliferator-activated receptor- γ coactivator α ; PPAR γ , proliferator-activated receptor γ ; AKT, protein kinase B; siRNA, small interfering RNA; SIRT1, sirtuin 1; TG, triglyceride; UPR, unfolded protein response; NAD, nicotinamide adenine dinucleotide; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; BCA, bicinchoninic acid; Pdk4, pyruvate dehydrogenase kinase 4, isoenzyme 4; Acox1, acyl-CoA oxidase 1; Ehhadh, enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase

Key words: erythropoietin, sirtuin 1, fibroblast growth factor 21, unfolded protein response, hepatic steatosis

lipophagy (14,15); however, the mechanism underlying the role of EPO in hepatic lipid metabolism, ER stress in particular, remains unknown.

Sirtuin 1 (SIRT1), a member of the sirtuin family of nicotinamide adenine dinucleotide (NAD⁺)-dependent protein deacetylases, regulates key aspects of lipid metabolism (16,17). SIRT1 can deacetylate protein kinase-like ER kinase (PERK) and inhibit the PERK-eukaryotic initiation factor 2 α (eIF2 α) axis of the UPR pathway, thereby suppressing hepatic steatosis in mice (18-20). Our previous study revealed that the NAD⁺/NADH ratio and SIRT1 activity increased in response to EPO in HepG2 cells *in vitro* (15). However, the role of SIRT1 in association with EPO in ER stress remains unclear. In addition, SIRT1-induced fibroblast growth factor 21 (FGF21) expression was found to play a critical role in controlling obesity and energy balance (21,22).

The aim of the present study was to investigate the effects of EPO on hepatic ER stress and FGF21 expression, and determine whether this mechanism involves SIRT1.

Materials and methods

Animal model. All animal studies were performed in accordance with the National Institutes of Health guidelines and were approved by the Nanjing University Medical School Institutional Animal Care and Use Committee. Male hepatocyte-specific SIRT1-deleted (SIRT1-LKO) mice and wild-type (WT, C57BL/6J) littermates (8 weeks old) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). The mice were housed under controlled temperature (20-22°C) and humidity (50%) conditions, with a 12-h light/dark cycle. All mice were given free access to high-fat diet (HFD; 60% calories from fat, 25% calories from carbohydrates and 15% calories from protein; Yangzhou University, Yangzhou, China) or standard chow (NC; 10% calories from fat, 75% calories from carbohydrates and 15% calories from protein; Yangzhou University), as described below. The mice were intraperitoneally injected with 3,000 U/kg of recombinant human EPO (Sunshine Pharmaceutical) or an equivalent volume of PBS every other day (n=6 per group). For the first experiment, SIRT1-LKO mice and WT littermates were fed HFD for 12 weeks, and then divided into two groups prior to treatment with EPO or PBS for 5 weeks. For the second experiment, C57BL/6J mice were treated with EPO or PBS for 7 or 14 days; the mice were given free access to NC and water. Finally, for the third experiment, C57BL/6J mice were fed HFD or NC for 12 weeks; the HFD group was intraperitoneally injected with EPO or PBS for 5 weeks. Lean littermate control mice were intraperitoneally injected with an equivalent volume of PBS. Body weight and fat were measured weekly (AccuFat-1050, MAG-MED). After 5 weeks, the mice were fasted for 8 h and underwent an intraperitoneal glucose tolerance test (IPGTT), as previously described (14). Glucose levels were determined from blood samples obtained via the tail vein at 0, 30, 60 and 120 min following glucose administration (1.5 g/kg). Subsequently, all the mice were deprived of food for 8 h prior to sacrifice for the collection of blood samples and liver tissues for further examination. After being weighed, fresh liver tissues were subjected to hematoxylin and eosin (H&E; Goodbio) and Oil Red O staining (Goodbio) (15).

For immunofluorescence, the sections were incubated with rabbit anti-protein disulfide isomerase (PDI) antibody (1:500; Cell Signaling Technology, Inc.; cat. no. 3501). For immunohistochemistry (IHC), the sections were incubated with rabbit anti-FGF21 antibody (1:200; Abcam; cat. no. ab66564) and quantitated by ImageJ plugin IHC profiler (National Institutes of Health), as previously described (23-25). Liver triglyceride (TG) content was measured using an ELISA kit according to the manufacturer's instructions (BioVision, Inc.). All samples were stored at -80°C for further analysis.

Cell culture. Primary hepatocytes were isolated from 8-12-week-old C57BL/6J mice (20-25 g) using a two-step perfusion method, and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), as described previously (26). For pharmacological studies, cells were treated with 40 U/ml EPO, 0.3 mmol/l palmitate (PA; Sigma-Aldrich; Merck KGaA) or 1 μ mol/l thapsigargin (Thp; Sigma-Aldrich; Merck KGaA) for 24 h. Where indicated, the cells were pre-treated with 40 μ mol/l resveratrol (Sigma-Aldrich; Merck KGaA) or 2 μ mol/l EX-527 (MedChem Express, LLC) for 1 h prior to EPO treatment. Primary hepatocytes isolated from EPO-treated and control mice were incubated with 1, 2 and 10 nmol/l human recombinant FGF21 (Abcam) for 4 h; vehicle treatment served as control.

Small interfering RNA (siRNA) transfection. Primary hepatocytes were transfected with siRNA against SIRT1 (GenePharma Co., Ltd.) using Lipofectamine[®] 3000 (Thermo Fisher Scientific, Inc.). PA and EPO were added to the cells at 36 h post-transfection. The siRNA sequences were as follows: SIRT1 siRNA, 5'-GAUGAAGUUGACCUCCUCA-3'; and negative control, 5'-UUCUCCGAACGUGUCACGUTT-3'.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from mouse liver tissue using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RT of RNA into cDNA was performed with the RT reagent kit (Takara Bio, Inc.), followed by qPCR analysis on an ABI StepOne Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using SYBR Green (Roche Diagnostics) at a final volume of 20 μ l, according to the manufacturer's protocol. The primers used were as follows: FGF21, forward 5'-CCTCCAGTTTGGGGGTCAAG-3' and reverse 5'-CTGGTTTGGGGAGTCCTTCT-3'; peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α), forward 5'-GTCCTTCCTCCATGCCTGAC-3' and reverse 5'-TAGCTGAGCTGAGTGTGGC-3'; FGF receptor 1 (FGFR1), forward 5'-GTGGAG AATGAGTATGGGAGC-3' and reverse 5'-GGATCTGGA CATACGGCAAG-3'; single-pass transmembrane protein β Klotho, forward 5'-ACGAGGGCTGTTTTATGTGG-3' and reverse 5'-CAGGTGAGGATCGGTAACTG-3'; acyl-CoA oxidase 1 (Acox1), forward 5'-TGCCATTCGATACAGTGTG-3' and reverse 5'-CAGGAGCGGGAAGAGTTTATAC-3'; pyruvate dehydrogenase kinase 4, isoenzyme 4 (Pdk4), forward 5'-GGATTACTGACCGCCTCTTTAG-3' and reverse 5'-GGGAGCTTTTCTACAGACTCAG-3'; enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase (Ehhadh), forward

5'-AGCTGTTTATGTACCTTCGGG-3' and reverse 5'-CTGCTTTGGGTCTGACTCTAC-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward 5'-TGGCCTTCCGTGTTTCTTAC-3' and reverse 5'-GAGTTGCTGTTGAAGTCGCA-3'. Expression was normalized to GAPDH and determined via the $2^{-\Delta\Delta C_q}$ method (27).

Western blot analysis. Liver tissues or cultured hepatocytes were lysed with radioimmunoprecipitation assay lysis buffer (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with protease inhibitor cocktail (Roche Diagnostics). Total protein concentration was measured using the bicinchoninic acid method (BCA protein assay kit; Thermo Fisher Scientific, Inc.). Subsequently, 20 μ g of each sample was separated via 8–15% Bis-Tris NuPAGE gels (Goodbio) and transferred to polyvinylidene difluoride membranes (Invitrogen; Thermo Fisher Scientific, Inc.). The membranes were blocked with 5% non-fat milk or bovine serum albumin, and incubated overnight at 4°C with specific antibodies. Antibodies against SIRT1 (rabbit monoclonal, cat. no. 9475, 1:1,000), PERK (rabbit monoclonal, cat. no. 3192, 1:1,000), phosphorylated (p)-eIF2 α (Ser51, rabbit monoclonal, cat. no. 3398, 1:1,000), eIF2 α (rabbit monoclonal, cat. no. 5324, 1:1,000) and GAPDH (rabbit monoclonal, cat. no. 5174, 1:2,000) were obtained from Cell Signaling Technology, Inc. The anti-FGF21 antibody (rabbit polyclonal, cat. no. ab66564, 1:1,000) and glucose-regulated protein 78 (GRP78; rabbit polyclonal, cat. no. ab21685, 1:1,000) were purchased from Abcam. The anti-p-PERK antibody (Thr981, rabbit polyclonal, cat. no. YP1055, 1:1,000) was obtained from ImmunoWay Biotechnology. The membranes were then washed and incubated with a secondary antibody (goat anti-rabbit IgG, ZSGB-BIO; OriGene Technologies, Inc.) at room temperature for 1 h. The antibody-antigen complexes were visualized by enhanced chemiluminescence (ECL; EMD Millipore). Band intensities were quantified using ImageJ software (National Institutes of Health). All antibodies were validated by the manufacturer.

FGF21 levels. According to the manufacturer's instructions, the levels of FGF21 in mouse serum and cell culture supernatant were determined using a FGF21 ELISA kit (cat. no. ab212160, Abcam). Briefly, after equilibrating all reagents at room temperature, 50 μ l of samples or standards were added to the wells, followed by the antibody cocktail. After 1 h of incubation, the wells were washed three times to remove unbound material. Subsequently, 100 μ l 3,3',5,5'-tetramethylbenzidine substrate was added to each well and incubated for 15 min at room temperature in the dark. The reaction was terminated by adding 50 μ l stop solution and the optical density was recorded at 450 nm.

Statistical analysis. All analyses were performed using SPSS software (version 22.0, IBM Corp.). All data are expressed as the mean $\pm$ standard error of the mean. Differences between multiple groups were determined by performing one-way ANOVA followed by the least significant difference or Dunnett's T3 post hoc test. Student t-tests were used to assess differences between two groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

EPO-induced suppression of hepatic steatosis and reductions in body weight are compromised by hepatic-specific SIRT1 deletion in HFD-fed mice. After 5 weeks of treatment, EPO-treated HFD-fed mice exhibited weight loss and reduced body fat compared with control mice (Fig. 1A and B). Additionally, IPGTTs revealed that the blood glucose levels were lower in EPO-treated mice compared with those in the PBS-treated group (Fig. 1C). Furthermore, histological analysis, including H&E and Oil Red O staining, demonstrated that EPO markedly alleviated hepatic steatosis in HFD-fed WT mice (Fig. 1D). Consistently, liver TG levels and liver weight were decreased in response to EPO treatment in the WT group (Fig. 1E and F). Compared with WT mice, these beneficial effects on body weight and hepatic steatosis were not observed in SIRT1-LKO mice. These results suggested a critical role for hepatic SIRT1 in mediating the protective effects of EPO in attenuating obesity and hepatic lipid accumulation.

EPO suppresses hepatic ER stress in a SIRT1-dependent manner. ER stress plays an important role in the development of hepatic steatosis (6). To investigate whether EPO alleviates metabolic ER stress, we measured the levels of the specific ER stress markers PDI and GRP78, and investigated PERK/eIF2 α signaling in the UPR pathway. In HFD-fed mice, EPO intervention notably inhibited the expression of PDI, GRP78, p-PERK and p-eIF2 α (Fig. 2A and B). *In vitro*, EPO-treated primary hepatocytes were incubated with PA or the ER stress inducer Thp. Treatment with PA or the ER stress agonist promoted the expression of GRP78, p-PERK and p-eIF-2 α , in parallel with an increase in TG content, suggesting that activation of ER stress may induce hepatic steatosis. Conversely, the upregulation of these ER stress markers and increased TG content induced by Thp and PA were suppressed by EPO (Figs. 3A and S1).

SIRT1, a potent regulator of energy metabolism and stress response, may play an important role in fatty liver and obesity (18,28). The present study investigated whether SIRT1 mediates the effects of EPO on hepatic ER stress and steatosis. As shown in Fig. 2B, no notable alterations in the expression of GRP78, p-PERK or p-eIF2 α were observed in SIRT1-LKO mice following EPO treatment. In addition, the effects of EPO on decreasing the protein expression levels of GRP78, p-PERK and p-eIF2 α were attenuated following SIRT1 inhibition by siRNA-mediated silencing in PA-treated primary hepatocytes (Figs. 3B and S2). Collectively, these findings indicate that SIRT1 may mediate the effects of EPO on alleviating hepatic ER stress and lipid accumulation.

EPO increases hepatic FGF21 expression. In view of the notable effects of EPO treatment on the liver and whole-body metabolism, it was hypothesized that a hepatokine may mediate the effects of EPO on alleviating hepatic lipid accumulation and obesity. Thus, the expression of FGF21, a hormone secreted by the liver that acts as a potent regulator of lipid metabolism, was analyzed (29). The results revealed that the mRNA and protein expression levels of FGF21 were increased in primary hepatocytes following treatment with 20–40 U/ml EPO (Fig. 4A and B), which was consistent with

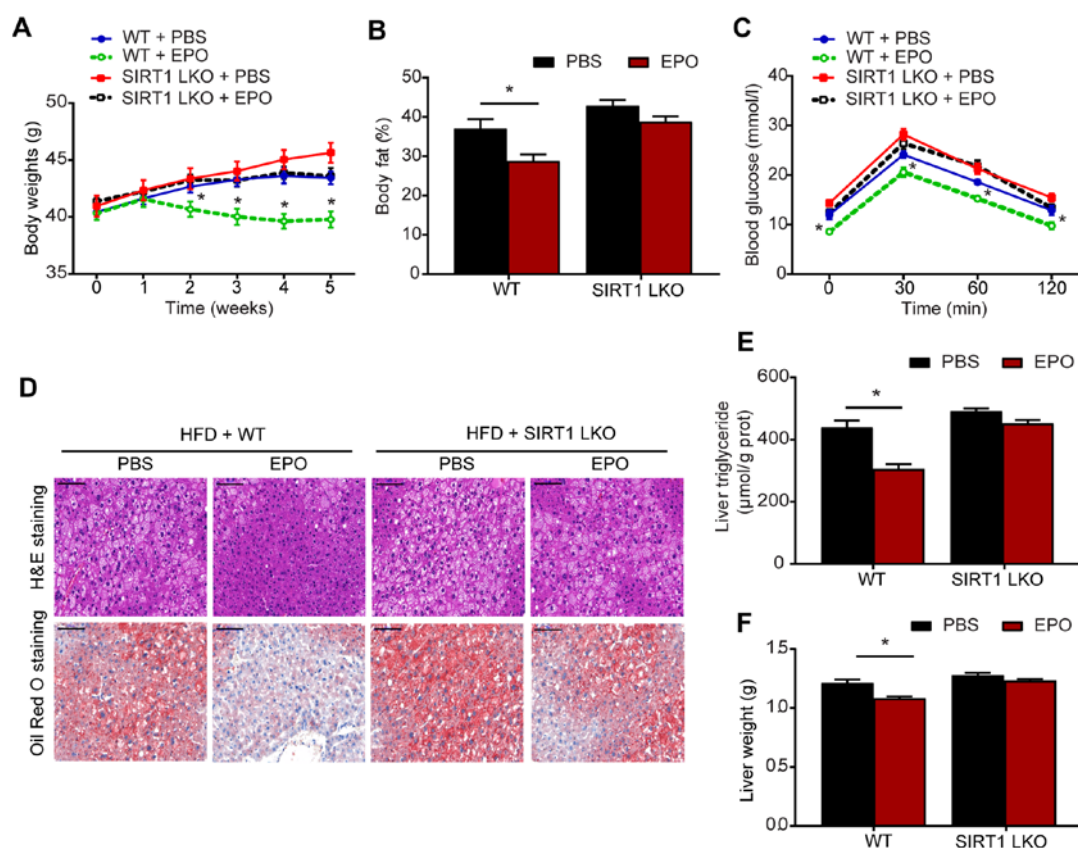


Figure 1. EPO-induced reductions in hepatic steatosis and body weight were compromised by hepatic specific-deletion of SIRT1 in HFD-fed mice. (A) Body weight, (B) body fat ratio and (C) IPGTTs were conducted following treatment. (D) H&E and Oil Red O staining (scale bar, 100 μ m), and analysis of (E) triglyceride (TG) content and (F) liver weight were performed on the livers of WT and SIRT1-LKO mice. Data are presented as the mean $\pm$ standard error of the mean (n=6/group, *P<0.05 vs. WT + PBS group). EPO, erythropoietin; SIRT1, sirtuin 1; HFD, high-fat diet; IPGTTs, intraperitoneal glucose tolerance tests; H&E, hematoxylin and eosin; WT, wild-type; SIRT1-LKO, hepatocyte-specific SIRT1-deleted.

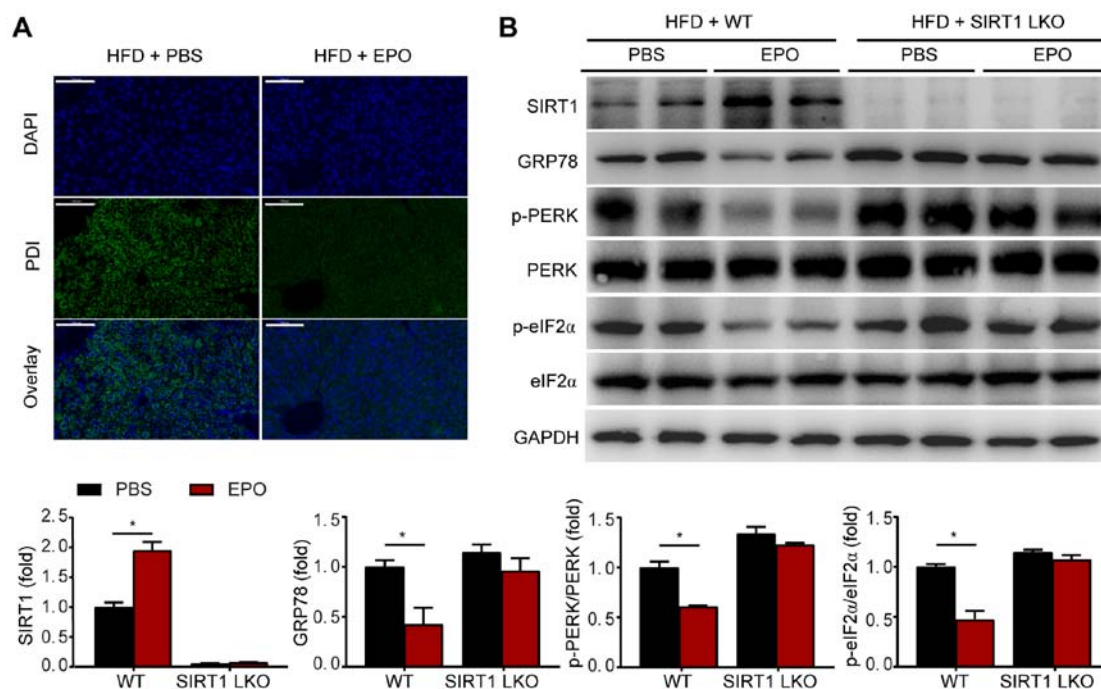


Figure 2. The effects of EPO on inhibiting hepatic ER stress were eliminated in SIRT1-LKO mice. (A) The ER stress marker PDI was detected by immunofluorescence staining in liver tissues of HFD-fed WT mice with or without EPO intervention. Green dots indicate PDI expression. Scale bar, 50 μ m. (B) Following EPO treatment, hepatic SIRT1, GRP78, p-PERK/PERK and p-eIF2 α /eIF2 α protein expression were determined by western blotting in the livers of EPO-treated WT and SIRT1-LKO mice. Data are presented as the mean $\pm$ standard error of the mean (n=6/group, *P<0.05 vs. WT + PBS group). EPO, erythropoietin; ER, endoplasmic reticulum; SIRT1-LKO, hepatocyte-specific SIRT1-deleted; WT, wild-type; HFD, high-fat diet; PDI, protein disulfide isomerase; GRP78, glucose-regulated protein 78; p-PERK, phosphorylation of PRKR-like endoplasmic reticulum kinase; p-eIF2 α , phosphorylation of eukaryotic translation initiation factor 2 α .

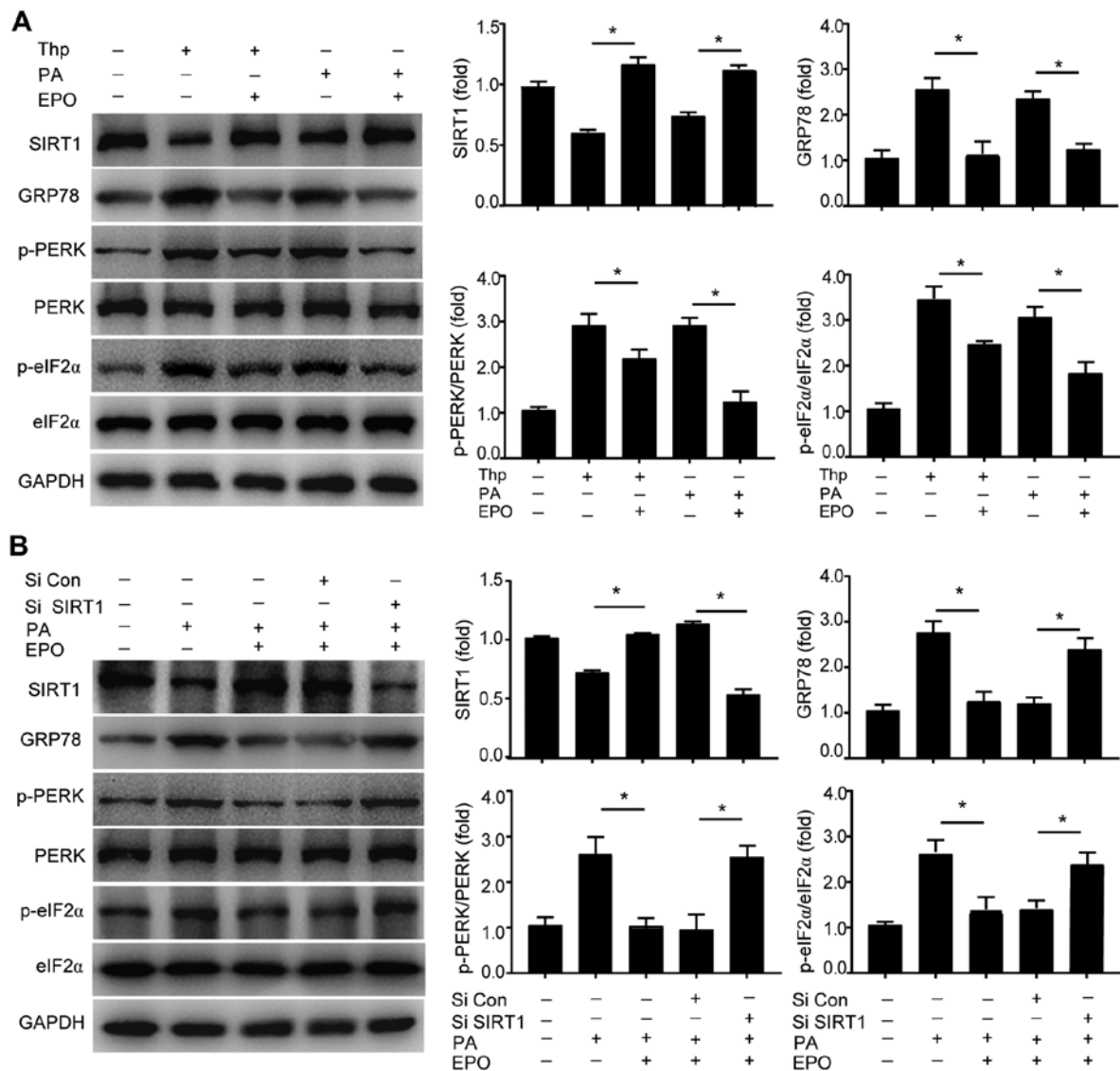


Figure 3. EPO inhibits lipid-induced hepatic ER stress in a SIRT1-dependent manner. The protein expression levels of SIRT1, GRP78, p-PERK/PERK and p-eIF2α/eIF2α protein expression were determined (A) in Thp- or PA-treated primary hepatocytes, and (B) in hepatocytes treated with SIRT1-targeting siRNA. Data are presented as the mean $\pm$ standard error of the mean ($n=3$ independent experiments, $P<0.05$). EPO, erythropoietin; ER, endoplasmic reticulum; SIRT1, sirtuin 1; GRP78, glucose-regulated protein 78; p-PERK, phosphorylation of PRKR-like endoplasmic reticulum kinase; p-eIF2α, phosphorylation of eukaryotic translation initiation factor 2α; Thp, thapsigargin; PA, palmitate; siRNA, small interfering RNA.

the alterations in FGF21 expression in cell culture (Fig. S3A). The results of PCR, western blotting and IHC revealed that short-term EPO treatment for 7 or 14 days in mice fed NC promoted FGF21 mRNA and protein expression in liver tissues (Fig. 4C-F), which was accompanied by increased levels of circulating FGF21 (Fig. S3B). Moreover, previous evidence indicated that SIRT1-mediated activation of FGF21 prevented liver steatosis and obesity (21). Therefore, whether SIRT1 is required for EPO-induced FGF21 expression was investigated. As shown in Fig. 5A, EPO induced the expression of SIRT1 and FGF21 in hepatocytes, mimicking the effects of the SIRT1 agonist resveratrol. Conversely, the SIRT1 inhibitor EX-527 suppressed the upregulated expression of FGF21 mediated by EPO. In addition, EPO treatment increased the expression levels of FGF21 in the circulation and liver tissues of HFD-fed WT mice, but this effect was mitigated in mice harboring a hepatic-specific deletion of SIRT1 (Figs. 5B and S3C).

Collectively, these results suggest that SIRT1 is required for EPO-stimulated FGF21 production in hepatocytes.

EPO partially restores sensitivity of hepatocytes to FGF21 in HFD-fed mice. An HFD diet was reported to suppress the expression of hepatic FGF21 receptors, including FGFR1 and β Klotho, and suggested that obesity occurs under FGF21-resistant conditions (30). In the livers of HFD-fed mice, EPO treatment partially restored the expression of the aforementioned receptors (Fig. 6A and B). Additionally, Acox1, Pdk4 and Ehhadh have been proposed as downstream target genes of the FGF21/PGC-1α axis associated with hepatic lipid β -oxidation (30,31). The mRNA expression levels of these genes were decreased in the livers of mice administered an HFD challenge. Conversely, EPO intervention restored the expression of PGC-1α, Acox1 and Ehhadh, but not Pdk4 (Fig. 6C and D). In addition, FGF21-induced phosphorylation

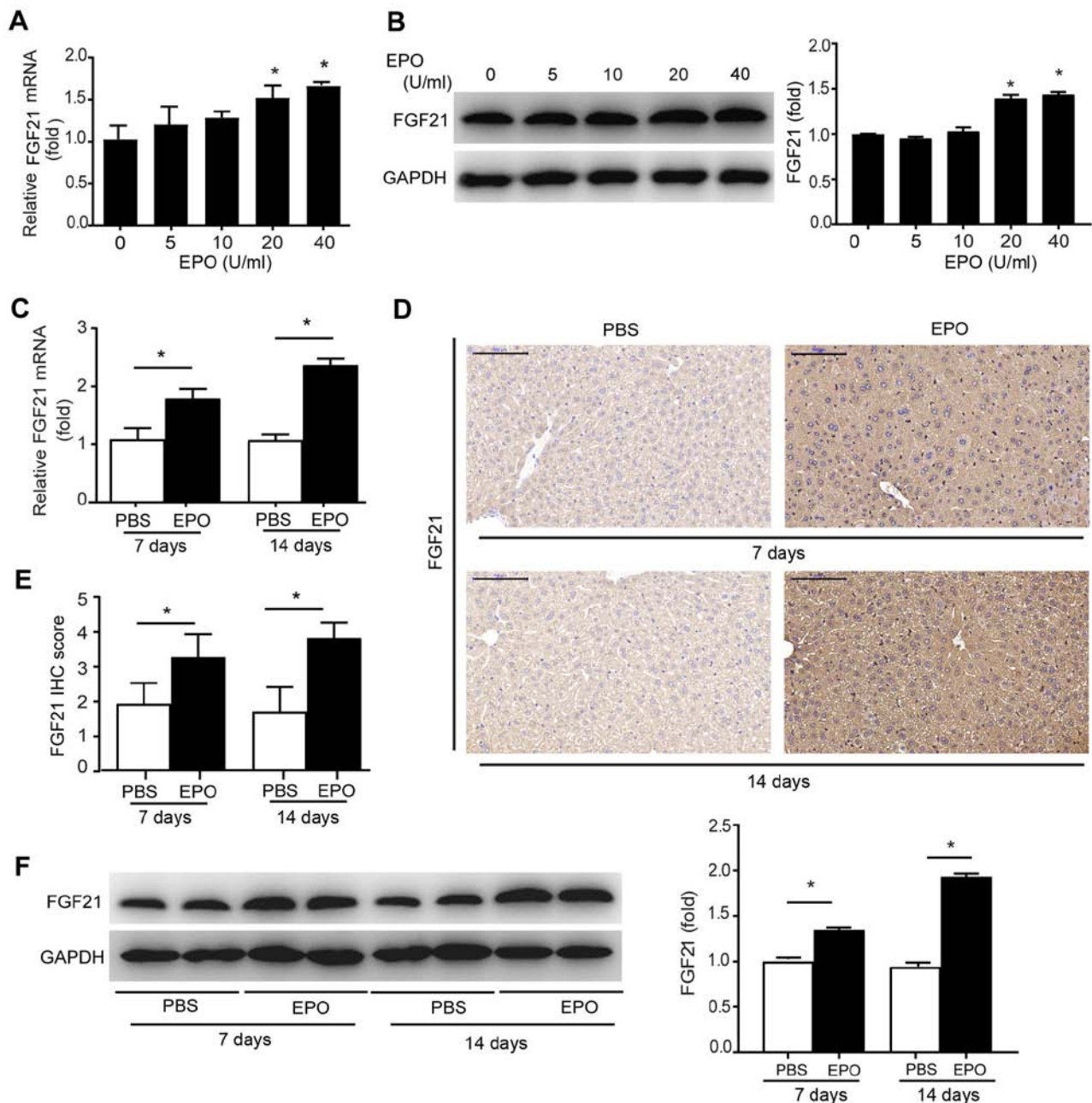


Figure 4. EPO induced hepatic FGF21 expression. (A) The mRNA and (B) protein expression levels of FGF21 were determined in primary hepatocytes treated with the indicated concentrations of EPO. Data are presented as the mean $\pm$ standard error of the mean ($n=3$ independent experiments, $^*P<0.05$, vs. 0 U/ml). (C) The mRNA expression levels of hepatic FGF21 were determined in mice treated with short-term EPO or PBS; FGF21 protein expression was (D) visualized and (E) quantified based on IHC, and (F) measured by western blotting. Scale bar, 100 μ m. Data are presented as the mean $\pm$ standard error of the mean ($n=6$ /group, $^*P<0.05$ vs. PBS group). EPO, erythropoietin; FGF21, fibroblast growth factor 21; IHC, immunohistochemistry.

of eIF2 α was inhibited in mouse hepatocytes isolated from HFD mice; however, FGF21 intervention enhanced eIF2 α phosphorylation (Fig. 6E). Collectively, these results demonstrated that EPO treatment enhanced FGF21 sensitivity in response to alterations in metabolism.

Discussion

EPO has been attracting increasing attention for its potential beneficial effects against the progression of obesity, diabetes and fatty liver disease. However, the underlying mechanism mediating these effects remains unclear. In the present study,

a novel mechanism by which EPO alleviates obesity-induced ER stress and hepatic steatosis in a SIRT1-dependent manner was proposed. Additionally, EPO was reported to serve as a positive regulator of hepatic FGF21 expression, further supporting the hypothesis that EPO may be a promising agent for the treatment of hepatic steatosis and obesity.

Hepatic ER stress, induced by pharmacological agents or metabolic dysregulation, promotes hepatic lipid accumulation by increasing lipogenesis (32). Genetic ablation studies revealed that the phosphorylation of eIF2 α , a key downstream target of PERK, exacerbates the progression of hepatic steatosis in mice subjected to pharmacologically induced ER stress (33).

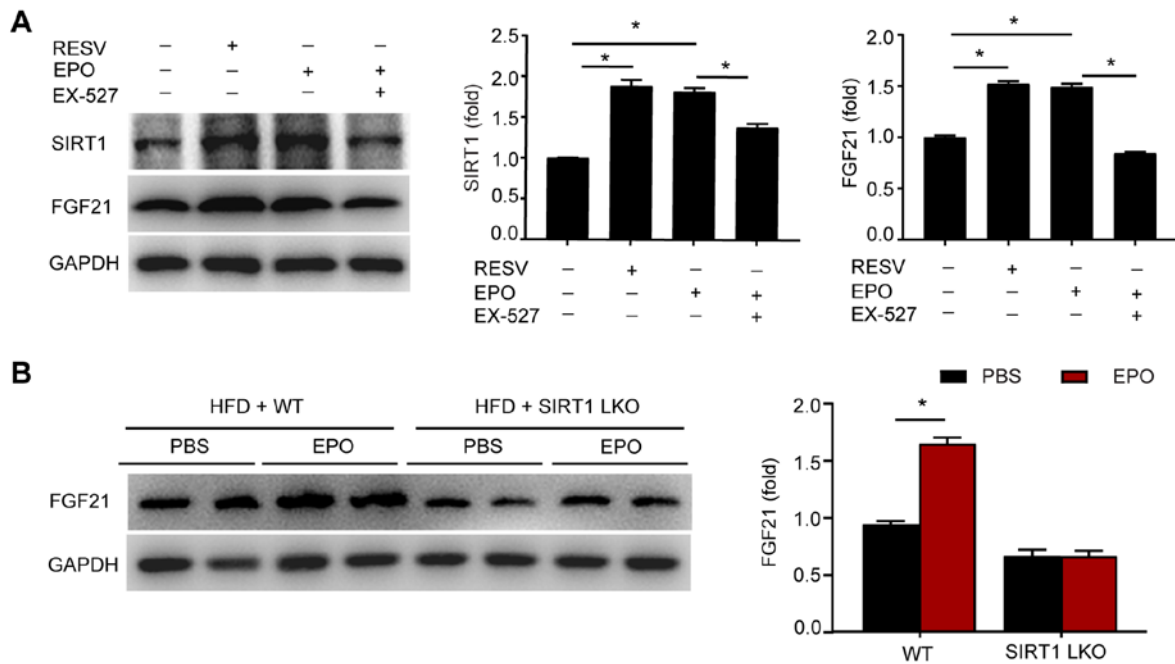


Figure 5. SIRT1 is required for EPO-induced FGF21 expression. (A) The protein expression levels of SIRT1 and FGF21 were determined by western blotting in primary hepatocytes exposed to resveratrol, EPO or EX527. Data are shown as the mean $\pm$ standard error of the mean (n=3 independent experiments, *P<0.05). (B) The protein expression levels of FGF21 were determined in the liver tissues of HFD-fed WT and SIRT1-LKO mice. Data are shown as the mean $\pm$ standard error of the mean (n=6/group, *P<0.05 vs. WT + PBS group). EPO, erythropoietin; SIRT1, sirtuin 1; FGF21, fibroblast growth factor 21; HFD, high-fat diet; WT, wild type; SIRT1-LKO, hepatocyte-specific SIRT1-deleted. RESV, resveratrol.

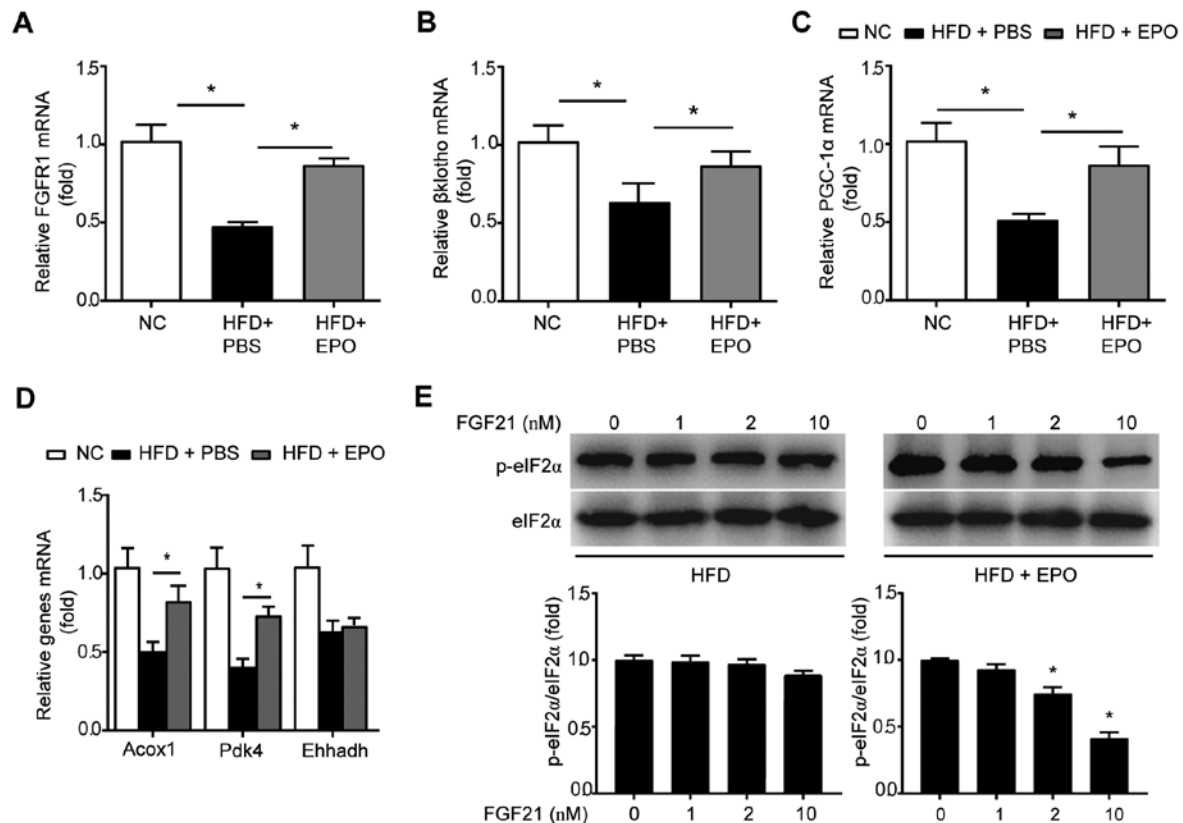


Figure 6. EPO partially restored FGF21 sensitivity in the hepatocytes of HFD-fed mice. The mRNA expression levels of FGF21 co-receptors (A) FGFR1, (B) β Klotho, (C) PGC-1 α and its target genes, including (D) Acox1, Pdk4 and Ehhadh were measured in liver tissues of HFD-fed mice following EPO treatment. Data are presented as the mean $\pm$ standard error of the mean (n=6/group, *P<0.05). (E) Primary hepatocytes, isolated from HFD-fed mice treated with PBS or EPO, were incubated with indicated dosage of recombinant FGF21, followed by western blotting to analyze p-eIF2 α /eIF2 α expression. Data are shown as the mean $\pm$ standard error of the mean (n=3 independent experiments, *P<0.05). EPO, erythropoietin; SIRT1, sirtuin 1; FGF21, fibroblast growth factor 21; HFD, high-fat diet; PGC-1 α , peroxisome proliferator-activated receptor- γ coactivator α ; Acox1, acyl-CoA oxidase 1; Pdk4, pyruvate dehydrogenase kinase 4, isoenzyme 4; Ehhadh, enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase; p-eIF2 α , phosphorylation of eukaryotic translation initiation factor 2 α .

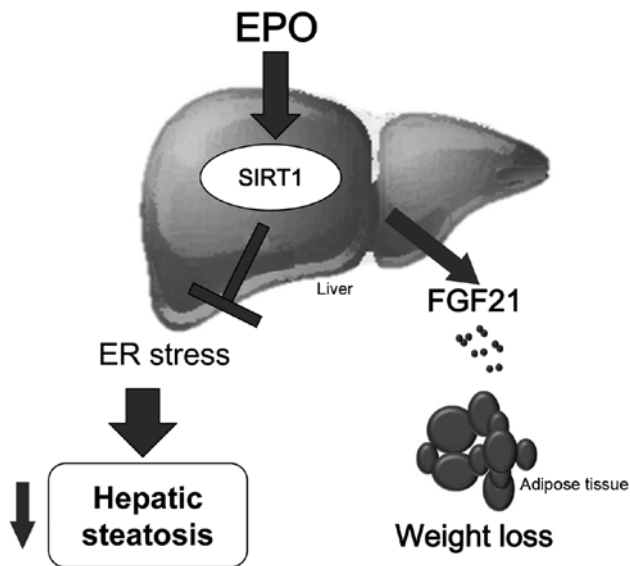


Figure 7. Model of the newly proposed mechanism whereby EPO alleviates hepatic endoplasmic reticulum stress and induces the expression of FGF21 in a SIRT1-dependent manner. Targeting of this mechanism may be of therapeutic value for hepatic steatosis and obesity. EPO, erythropoietin; SIRT1, sirtuin 1; FGF21, fibroblast growth factor 21.

Conversely, transgenic mice with inactivated eIF2 α via dephosphorylation were protected from HFD-induced hepatic steatosis (34). In the present study, in addition to the protective effects of EPO against weight gain and fat accumulation, EPO was observed to decrease lipid content and alleviate ER stress in the livers of HFD-fed mice and PA-induced hepatocytes. These observations were associated with decreases in the expression levels of ER stress markers, including PDI, GRP78, p-PERK and p-eIF2 α . Consistently, previous studies have reported the ability of EPO to protect rats against cardiac dysfunction and nephrotoxicity by attenuating intracellular ER stress (35,36). Furthermore, SIRT1 overexpression was proposed to attenuate lipid-induced ER stress and hepatic accumulation by decreasing mammalian target of rapamycin complex 1 activity (18,37). Our previous study reported that EPO was able to increase hepatic SIRT1 activity *in vitro* (15). The results of the present study suggested the presence of *in vivo* cross-talk between EPO and SIRT1 in liver tissue. As the alleviating effect of EPO on ER stress and fatty liver was abrogated in SIRT1-LKO mice, SIRT1 may regulate the effects of EPO in the liver. Additionally, SIRT1 loss-of-function approaches in hepatocytes via siRNA further indicated that SIRT1 is required for EPO to attenuate hepatic ER stress and lipid deposition. Collectively, these findings revealed a novel mechanism through which EPO alleviates ER stress in hepatic steatosis in a SIRT1-dependent manner.

Furthermore, the effects of EPO on alleviating metabolic dysregulation were eliminated in SIRT1-LKO mice. Previous studies reported that hepatic SIRT1 markedly induced FGF21 expression and secretion to control whole-body energy metabolism (21,38). The hepatokine FGF21 has been considered as a promising therapeutic agent that acts by promoting hepatic fatty acid oxidation, the browning of white adipose tissue and the thermogenesis of brown adipose tissue (39-41). The present study suggested that EPO could increase the expression of

hepatic FGF21 *in vivo* and *in vitro*. However, SIRT1 deficiency (genetic or pharmacologically induced) inhibited the effects of EPO on the induction of FGF21, which was accompanied by diminished effect on increased expression of FGF21-targeted PGC-1 α . These results suggested that SIRT1-induced FGF21 expression may contribute to the effects of EPO on alleviating hepatic steatosis and obesity. Of note, obese animals or human subjects, or those with fatty liver, had elevated FGF21 levels, indicating that the function of FGF21 is impaired under conditions of fat accumulation (30,42). The expression of FGFR1 and β Klotho, which serve as co-receptors of FGF21, was down-regulated by HFD feeding (43). Additionally, EPO treatment restored the expression of these receptors, which was accompanied by the upregulation of FGF21/PGC-1 α -axis target genes, including Acox1 and Pdk4. In addition, eIF2 α is a molecular target of FGF21 in the regulation of ER stress in the liver (44); the present study demonstrated that eIF2 α phosphorylation in response to FGF21 in mouse hepatocytes was restored by EPO treatment, compared with PBS-treated obese mice. As regards the feedback mechanism underlying the effects of FGF21 on lipid metabolism related to ER stress (45), further investigation is required to determine the role of FGF21 in association with the effects of EPO on alleviating ER stress. Collectively, the results of the present study indicated that EPO treatment induced FGF21 expression and restored its sensitivity under conditions of obesity, contributing to improved systemic metabolism.

In summary, the results of the present study demonstrated that EPO ameliorated hepatic steatosis and obesity by inducing SIRT1-mediated inhibition of ER stress and promoting FGF21 expression (Fig. 7). These findings may improve our understanding of this mechanism and provide more experimental evidence on the therapeutic value of EPO in fatty liver and obesity.

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Availability of data and materials

The datasets generated and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

TH contributed to the study design, data acquisition and interpretation, drafting of the article, and revision of the manuscript. ZG contributed to the study design, data acquisition, data analysis, and the drafting and revision of the manuscript. BZ and RM contributed to the acquisition and analysis of the data and the drafting of the manuscript. YB contributed to the study design, data interpretation, and the drafting and revision of the manuscript. DZ contributed to the study design, data interpretation, and the revision of the manuscript. All authors have read and approved the final version of this manuscript for publication.

Ethics approval and consent to participate

All animal experiments were approved by the Nanjing University Medical School Institutional Animal Care and Use Committee, and were performed in accordance with the National Institutes of Health guidelines.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests associated with this publication.

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