ORIGINAL RESEARCH COMMUNICATION

Isosteviol Protects Free Fatty Acid- and High Fat Diet-Induced Hepatic Injury *via* Modulating PKC- β /p66Shc/ROS and Endoplasmic Reticulum Stress Pathways

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Abstract

Aims: Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver diseases. However, there are no approved pharmacotherapies for the treatment of NAFLD other than managing life style and controlling diets. Extensive studies have demonstrated that multiple mechanisms are involved in free fatty acid (FFA)- and high fat diet (HFD)-induced hepatic injury, including mitochondrial dysfunction, activation of oxidative stress and endoplasmic reticulum (ER) stress, and lysosome dysfunction. A previous study reported that Isosteviol (ISV), a derivative of stevioside, prevents HFD-induced hepatic injury. However, the underlying mechanisms remain unclear.

Results: In this study, we examined the potential cellular/molecular mechanisms underlying ISV-mediated protective effect against FFA-/HFD-induced hepatic lipotoxicity by using both *in vitro* primary rat hepatocytes and the *in vivo* rat NAFLD model. The results indicated that ISV inhibits FFA-/HFD-induced hepatic injury *via* reducing oxidative and ER stress. Specifically, ISV inhibited the expression, activation, and mitochondrial translocation of Src-homology-2-domain-containing transforming protein 1 (p66Shc), an adapter protein that mediates oxidative stress-induced injury and is a substrate of protein kinase C- β (PKC- β), *via* inhibition of PKC- β activity. However, ISV had no effect on the expression and activity of peptidyl-prolyl cis-trans isomerase and serine/threonine protein phosphatase 2A, isomerase and phosphorylase of p66Shc. In addition, ISV also inhibited FFA-induced ER stress and decreased ER-mitochondrial interaction.

Innovation and Conclusion: We first identified that ISV prevents FFA-/HFD-induced hepatic injury through modulating PKC- β /p66Shc/oxidative and ER stress pathways. ISV represents a promising therapeutic agent for NAFLD in the future. *Antioxid. Redox Signal.* 30, 1949–1968.

Keywords: Isosteviol, free fatty acid, NAFLD, ROS, ER stress

Introduction

NONALCOHOLIC FATTY LIVER DISEASE (NAFLD), defined by the presence of hepatic steatosis (the presence of fat in the liver parenchyma without inflammation) in the absence of excess alcohol consumption, is a significant global health problem. There was a rapid increase of the prevalence of obesity and metabolic syndromes during the past decade (41, 52).

Unhealthy diets such as those rich in fructose, trans-fatty acids, and saturated fats are believed to be associated with the development of NAFLD. Increase of free fatty acids (FFAs) and accumulation of hepatic triglycerides (TGs) are major features of NAFLD and commonly occur in the setting of insulin resistance (IR) and obesity. Extensive studies have been done to identify the potential underlying mechanisms associated with NAFLD disease progression. The major

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Innovation

At present, no approved pharmacotherapies for the treatment of nonalcoholic fatty liver disease (NAFLD), a significant global health problem, are available. This study provides novel mechanistic evidence for Isosteviol-mediated protective effects against NAFLD. Isosteviol prevented free fatty acid-/HFD-induced hepatocyte injury through inhibition of protein kinase C- β (PKC- β) activation and expression, p66Shc mitochondria translocation, lipid accumulation, oxidative stress, endoplasmic reticulum (ER) stress, and ER-mitochondrial interaction. Our findings also indicate that Isosteviol has more profound effects than Ruboxistaurin, a specific inhibitor of PKC- β , on preventing hepatic lipotoxicity. Isosteviol represents a new promising therapeutic agent for NAFLD through modulating PKC- β /p66Shc/oxidative and ER stress pathways.

contributors identified so far include inflammatory cytokines, endoplasmic reticulum (ER) stress, and mitochondrial dysfunction secondary to nutrient excess and oxidative stress (14). Palmitic acid (PA) has been well characterized as a mediator of hepatic lipotoxicity (23, 49). The mechanism of PA-induced hepatocyte death is linked to an increase of reactive oxygen species (ROS) (19, 40), disruption of mitochondrial function (20), and activation of ER stress (32), c-Jun N-terminal kinase (JNK) (9), and Bax/Bcl-2 signaling pathways (30).

Although NAFLD is well recognized as the most common cause of liver disease, there are currently no approved pharmacotherapies. Drugs hoped to be effective are obeticholic acid, elafibranor, glucagon-like peptide-1 analogues, and C-C chemokine receptor types 2/5 antagonists (44). Recently, it has been demonstrated that several natural compounds, such as Isosteviol (4 α ,8 β ,13 β -13-methyl-16-oxo-17-nor-kauran-18-oic acid, ISV), have promising effects on the prevention of NAFLD (10).

ISV is an acid hydrolysate of stevioside, the major component of *Stevia rebaudiana*, and is used as a sweetener. Stevioside and its metabolites have been shown to have varieties of pharmacological activities such as antidiabetic and anti-hyperlipidemia effects (51). However, stevioside not only has toxic and mutagenic effects but also has very low oral bioavailability (31). Studies from our group and others have shown that ISV inhibits oxidative stress and reduces ROS levels in a rat focal cerebral ischemia injury model (50). ISV also has promising effects against high fat diet (HFD)-induced hepatic injury and IR, but it has much less toxicity (27).

Previous studies reported that mitochondrial translocation of the Src-homology-2-domain-containing transforming protein 1 (p66Shc), in response to a variety of proapoptotic stimuli, represents a key event of oxidative stress-mediated ROS production. Recent studies further showed that p66Shc-mediated signaling contributes to the oxidative stress-induced apoptosis and the progression of NAFLD (18a, 25, 28). However, the mechanisms by which ISV prevents FFA-/HFD-induced hepatic lipotoxicity remain to be identified and are the focus of this study.

Results

ISV inhibited HFD-induced hepatic injury in rat NAFLD models

It has been reported that ISV has protective effects against HFD-induced hyperglycemia and dyslipidemia (51). To determine whether ISV has any effect on HFD-induced hepatic steatosis and dysregulation of lipid metabolism, HFD-fed rat NAFLD models were used. Rats were fed with HFD or normal diet (ND). After 4 weeks, HFD-fed rats were further divided into three groups, HFD, HFD+ISV (10 mg/kg), and HFD+ISV (20 mg/kg), and treated with control or ISV for an additional 4 weeks. The body weight of HFD-fed rats increased more quickly (Fig. 1A) and the food intake of the HFD-fed rats decreased slightly after 3 weeks (Fig. 1B). There is no significant difference between all groups. As shown in Figure 1C, treatment with 20 mg/kg ISV inhibited HFD-induced increase of rat liver weight. The blood glucose levels did not change after HFD feeding with or without ISV treatment (Fig. 1D).

HFD-induced liver injury, as indicated by upregulation of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), was reduced by ISV (Fig. 1E). HFD-induced upregulation of TG, total cholesterol (TC), and low-density lipoprotein (LDL) was also inhibited by ISV at 20 mg/kg (Fig. 1F). As shown in Figure 1G, HFD-induced hepatic steatosis was significantly reduced by ISV, especially in the ISV 20 mg/kg group. In addition, ISV at 20 mg/kg also reversed HFD-induced increase in FFAs (Fig. 1H), suppression of superoxide dismutase (SOD) activity (Fig. 1I), and increase of malondialdehyde (MDA) (Fig. 1J).

ISV inhibited PA-induced apoptosis and lipid deposition in hepatocytes

PA-albumin complex was prepared by using a saponification technique by complexing PA (C16:0) to a fatty-acid free bovine serum albumin (39). Rat primary hepatocytes were treated with different amounts of PA-albumin complex,

FIG. 1. Effect of ISV on HFD-induced liver injury in rats. Rats were fed with regular rodent diet or HFD for 4 weeks and then the HFD-fed group rats were treated with different doses of ISV (0, 10, or 20 mg/kg) by oral gavage daily for 4 weeks. (A) Rat body weight. (B) Food intake. (C) Liver weight. (D) Blood glucose. (E) The enzyme activities of ALT and AST in the serum. (F) The serum lipid levels. (G) Representative images of H&E staining of the liver tissues from each treatment group are shown. (H) Hepatic FFAs levels normalized with total protein amount. (I) Hepatic SOD activity normalized with total protein amount. (J) Hepatic MDA levels normalized with total protein amount. The values were present with mean $\pm$ SD. $N=6-8$ rats per group. Statistical significance compared with control diet group or HFD-fed group, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and NS, no significance. ALT, alanine aminotransferase; AST, aspartate aminotransferase; FFA, free fatty acid; H&E, hematoxylin and eosin; HDL, high-density lipoprotein; HFD, high fat diet; ISV, Isosteviol; LDL, low-density lipoprotein; MDA, malondialdehyde; SD, standard deviation; SOD, superoxide dismutase; TC, total cholesterol; TG, triglyceride. Color images are available online.

0.25, 0.5, and 1 mM, for 24 h. The apoptotic cells were detected by Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining followed by flow cytometry. Consistent with our previous findings, PA significantly induced apoptosis in rat primary hepatocytes at a dose of 1 mM; whereas 0.25 and 0.5 mM of PA only slightly induced hepatocyte apoptosis (data not shown) (49). Therefore, 1 mM PA was used for all *in vitro* studies.

To determine the effect of ISV on PA-induced hepatocyte apoptosis, rat primary hepatocytes were treated with PA (1 mM) with different concentrations of ISV (0, 1, 3, and 10 μ M) for 24 h. As shown in Figure 2A, ISV dose dependently reduced the number of Annexin V-FITC and PI-positive hepatocytes. To determine the effect of ISV on PA-induced hepatocyte injury, we measured lactate dehydrogenase (LDH) leakage and Caspase-3 activities. As shown in Figure 2B and C, PA-induced increase of LDH leakage and Caspase-3 activities were inhibited by ISV in a dose-dependent manner in rat primary hepatocytes.

Similarly, PA-induced decrease of cell viability and increase of Caspase-3 activity were also inhibited in human

HepG2 cells (Supplementary Fig. S1A, B). To further determine the effect of ISV on PA-induced intracellular lipid accumulation, rat primary hepatocytes were stained with Nile red and analyzed by flow cytometry and fluorescence microscopy after treatment with PA and ISV for 24 h. As shown in Figure 2D and E, PA-induced intracellular lipid accumulation was inhibited by ISV, especially at the 10 μ M concentration. Similar results were obtained in HepG2 cells (Supplementary Fig S1C, D).

ISV inhibited PA-induced mitochondrial dysfunction

Maintenance of mitochondrial normal function is critical to hepatic lipid homeostasis. To identify the potential cellular mechanisms by which ISV prevents PA-induced hepatic injury, we measured mitochondrial transmembrane potential (MTP) and cytosolic cytochrome *c* contents. As shown in Figure 3A, PA significantly induced MTP collapse, as indicated by a decrease of JC-1 (5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolylcarbocyanine iodide) aggregates and an increase of JC-1 monomers, which were inhibited by ISV in a dose-dependent manner. Similar results were found in

FIG. 2. Effect of ISV on PA-induced apoptosis and lipid accumulation in rat primary hepatocytes. Rat primary hepatocytes were treated with PA (1 mM) and different concentrations of ISV (0, 1, 3, and 10 μ M) for 24 h. (A) Hepatocyte apoptosis was detected by annexin V-FITC and PI staining followed by flow cytometry. (B) The relative amount of LDH released to culture medium. (C) The relative Caspase-3 activity. (D) Intracellular lipid content was stained with Nile red and measured by flow cytometry. Representative flow cytometry diagrams from three independent experiments are shown. (E) Representative fluorescence microscope images of Nile red staining. The values are expressed as mean $\pm$ SEM of three independent experiments. Statistical significance compared with control group or PA group, * p < 0.05, ** p < 0.01, *** p < 0.001, and NS, no significance. FITC, fluorescein isothiocyanate; LDH, lactate dehydrogenase; PA, palmitic acid; PI, propidium iodide; SEM, standard error of the mean. Color images are available online.

HepG2 cells (Supplementary Fig. S1E). Although ISV had no effect on PA-induced increase of mitochondrial mass, it significantly reduced PA-induced cytochrome *c* release (Fig. 3B, C), an indicator of perturbation of mitochondrial membrane stability.

ISV inhibited PA-induced oxidative stress in hepatocytes

Activation of oxidative stress is a well-characterized mechanism of PA-induced hepatic lipotoxicity. The increase of intracellular ROS levels results in intracellular lipid peroxidation, induction of inflammatory mediators, protein damage, and hepatocyte apoptosis. To determine the effect of ISV on PA-induced oxidative stress in hepatocytes, rat primary hepatocytes were treated with PA (1 mM) with or without ISV for 24 h. The intracellular ROS levels were measured by 2',7'-dichlorofluorescein (DCF) staining followed by flow cytometric analysis. As shown in Figure 3D, PA-induced increase of intracellular ROS levels was reduced by ISV in a dose-dependent manner.

Similar results were obtained in HepG2 cells (Supplementary Fig. S1F). In addition, as shown in Figure 3E, PA-induced increase of MDA, a marker for oxidative stress, was also inhibited by ISV. ISV also reversed PA-induced reduction of SOD activity. Activation of oxidative stress is associated with an increase of lipid peroxidation. As shown in Figure 3E, PA-induced increase of the intracellular levels of 4-HNE, a product of lipid peroxidation, was also inhibited by ISV.

Previous studies reported that p66Shc is a key molecule that mediates oxidative stress-dependent tissue damage, including hepatocyte damage, in response to metabolic injuries (28). p66Shc is a cytosolic protein and is translocated into mitochondria under oxidative stress conditions. To determine whether p66Shc is involved in the ISV-mediated protective effect against PA-induced hepatic injury, we measured the protein levels of p66Shc in both cytosol and mitochondria. As shown in Figure 3F, ISV (10 μ M) inhibited PA-induced increase of p66Shc in both cytosol and mitochondria.

ISV inhibited PA-induced activation of protein kinase C- β in hepatocytes

Previous studies reported that the mitochondrial translocation of p66Shc is regulated by protein kinase C- β (PKC- β), peptidyl-prolyl cis-trans isomerase NIMA-interacting 1 (Pin1), and serine/threonine protein phosphatase 2A (PP2A)

(55). To further identify the signaling pathways involved in ISV-mediated inhibition of p66Shc expression and mitochondrial translocation, the protein expression levels of p-PKC- β , PKC- β , PP2A, and Pin1 in cytosol and membrane-bound PKC- β were measured by Western blot analysis after treatment with PA, with or without ISV, in rat primary hepatocytes for 24 h. As shown in Figure 4A and Supplementary Figure S2A, PA-induced upregulation of cytosolic p-PKC- β and PKC- β and membrane PKC- β was inhibited by ISV.

We further measured the direct effect of ISV on the kinase activity of PKC- β by using a luciferase reporter assay, as described in the Materials and Methods section. As shown in Figure 4B, ISV dose dependently inhibited PKC- β activity. The IC₅₀ of ISV on PKC- β was 7.4 μ M, which was much higher than that of Ruboxistaurin (RBX) (IC₅₀ = 12.7 nM), a PKC- β selective inhibitor used as a positive control. However, ISV had no effect on protein levels of PP2A and Pin1 (Supplementary Fig. S3A) and the enzyme activity of PP2A (Supplementary Fig. S3B). To further determine whether inhibition of PKC- β activity is a key mechanism underlying the ISV-mediated protective effect against PA-induced hepatic toxicity, we compared the effect of ISV with RBX on PA-induced hepatocyte apoptosis.

Even though ISV at 10 μ M had a similar inhibition of PKC- β activity as RBX at 20 nM, ISV had a more profound effect on PA-induced apoptosis in hepatocytes (Fig. 4C). Both ISV (10 μ M) and RBX (20 nM) inhibited PA-induced Caspase-3 activation and LDH leakage in rat primary hepatocytes (Supplementary Fig. S2B, C). Moreover, ISV at 10 μ M had a more profound inhibition of PA-induced intracellular lipid accumulation and p66Shc protein expression than RBX (20 nM) (Fig. 4D, E). However, ISV and RBX had a similar effect on PA-induced phosphorylation and mitochondrial translocation of p66Shc (Fig. 4E, F). These results suggest that modulation of p66Shc expression and intracellular translocation represent key cellular/molecular mechanisms underlying the ISV-mediated protective effect against PA-induced hepatic lipotoxicity.

Knocking down p66Shc protected hepatocytes from PA-induced injury

To further confirm the role of p66Shc in PA-induced hepatic injury, gene-specific lentiviral shRNAs were designed to knock down the expression of p66Shc. As shown in Figure 5A and Supplementary Figure S4A, both shRNA #1 and

FIG. 3. Effect of ISV on PA-induced mitochondrial dysfunction and oxidative stress. Rat primary hepatocytes were treated with PA (1 mM) and different concentrations of ISV (0, 1, 3, and 10 μ M) for 24 h. (A) The MTP was measured by JC-1 staining followed by flow cytometry. Representative flow cytometry diagrams of three independent experiments are shown. (B) The mitochondrial mass was measured by using MitoTracker Green FM. The relative amount of each treatment group to control group was calculated. (C) Representative immunoblot images of cytosolic cytochrome *c*, β -actin, and Cox4i1 are shown. (D) ROS levels were measured by flow cytometry to detect intracellular fluorescence intensity of DCF. (E) Relative amounts of 4-HNE, SOD, and MDA were measured as described in the Materials and Methods section. (F) Representative immunoblot images of p66Shc in cytosol and mitochondria. Cox4i1 and Actin were used as loading control of mitochondrial and cytosolic protein, respectively. JC-1 aggregates and high DCF intensity cells ratios were calculated, respectively, based on the mean $\pm$ SEM of three independent experiments. Relative protein levels of Cytochrome *c* and p66Shc were calculated based on the mean $\pm$ SD of three independent experiments. Statistical significance compared with control or PA group, * p < 0.05, ** p < 0.01, *** p < 0.001, and NS, no significance. Cox4i1, cytochrome *c* oxidase subunit 4i1; DCF, 2',7'-dichlorofluorescein; JC-1, 5,5,6,6-tetrachloro-1,1,3,3-tetraethylbenzimidazolylcarbocyanine iodide; MTP, mitochondrial transmembrane potential; p66Shc, Src-homology-2-domain-containing transforming protein 1; ROS, reactive oxygen species; SOD, superoxide dismutase.

shRNA #2 inhibited p66Shc mRNA and protein expression by 80%–90% in rat primary hepatocytes. Nile red staining analysis indicated that PA-induced intracellular lipid accumulation was reduced by both shRNAs (Fig. 5B). Similarly, PA-induced apoptosis, oxidative stress, collapse of MTP, and

cytochrome *c* release were all inhibited by p66Shc shRNAs (Fig. 5C and Supplementary Fig. S4C). To further determine the correlation of p66Shc expression in human NAFLD, immunohistochemical staining of p66Shc and PKC- β was performed with human liver tissue chips from NAFLD

A

B

C

D

E

F

FIG. 4. The role of PKC- β activation in PA-induced hepatic injury. (A) Rat primary hepatocytes were treated with PA (1 mM) with or without ISV (3 or 10 μ M) for 24 h. The protein levels of cytosolic phosphorylated PKC- β (p-PKC- β) and total PKC- β (t-PKC- β) were determined by Western blot analysis. Representative immunoblot images are shown. β -Actin was used as loading control. Relative protein levels of p-PKC- β and t-PKC- β were calculated based on the mean $\pm$ SD of three independent experiments. Statistical significance compared with control or PA group, * p < 0.05, ** p < 0.01. (B) The effect of ISV and RBX on PKC- β enzyme activity was measured by using an *in vitro* luciferase assay as described in the Materials and Methods section. The IC_{50} of ISV and RBX were calculated by using Sigma plot software. (C–F) Rat primary hepatocytes were treated with PA (1 mM) with vehicle control, ISV (10 μ M), or RBX (20 nM) for 24 h. (C) The apoptotic cells were measured by flow cytometry as described in the Materials and Methods section. (D) The intracellular lipid accumulation was measured by Nile red staining and flow cytometry. Annexin V positive cells ratio and Nile red fluorescence intensity were calculated, respectively, based on the mean $\pm$ SEM of three independent experiments. (E, F) Representative immunoblot images of phosphorylated p66Shc (p-p66Shc) and total p66Shc (t-p66Shc) in total cell lysate, cytosol, and mitochondria are shown. β -Actin and Cox4i1 were used as loading control for cytosolic and mitochondrial protein, respectively. Relative protein levels of p-p66Shc and p66Shc are presented based on the mean $\pm$ SD of three independent experiments. Statistical significance compared with control or PA group, * p < 0.05, ** p < 0.01, *** p < 0.001, and NS, no significance. PKC- β , protein kinase C- β ; RBX, Ruboxistaurin. Color images are available online.

patients and normal controls purchased from AlenaBio Biotechnology Co., Ltd. (Xi'an, China). As shown in Supplementary Figure S4E, both p66Shc and PKC- β were upregulated in NAFLD patients, suggesting that both p66Shc and PKC- β are involved in hepatic lipotoxic injury. Rat hepatocytes were transfected with p66Shc or p66Shc (S36D) phosphomimetic mutant plasmid (in which Ser36 was mutated to Asp). PA increased ROS and induced LDH leakage not only in p66Shc-transfected hepatocytes but also in p66Shc (S36D)-transfected hepatocytes (Fig. 5D, E).

ISV inhibited the effect of PA on p66Shc-transfected hepatocytes, but not in p66Shc (S36D)-transfected hepatocytes. Therefore, overexpression of the p66Shc (S36D) phosphomimetic mutant partially abrogated the protective effect of Isosteviol. It has been reported that p66Shc expression was regulated by p53 (42). To further identify the regulatory mechanism of the p66Shc expression, we examined the expression of p53 after PA treatment. As shown in Supplementary Figure S5A and S5B, PA increased p53 transcription in rat primary hepatocytes. ISV had no effect on the transcription of p53 and cytosolic protein levels but decreased nuclear p53 protein level. Reduction of p53 translocation might be one of the mechanisms underlying ISV-induced decrease of p66Shc expression; however, the exact mechanism remains to be identified.

Knocking down PKC- β inhibited PA-induced hepatic injury in rat primary hepatocytes

As shown in Figure 4, PKC- β plays a very important role in regulating p66Shc phosphorylation and mitochondrial translocation. To further delineate the role of PKC- β in PA-induced hepatic injury, lentiviral shRNAs targeting PKC- β were used to knock down PKC- β in rat primary hepatocytes. As shown in Figure 6A and Supplementary Figure S4B, both shRNA #1 and shRNA #2 inhibited PKC- β mRNA and protein expression by 80%–90%. PA-induced intracellular lipid deposition was also inhibited by PKC- β shRNA. Combination of PKC- β shRNA with ISV (10 μ M), not RBX, synergistically inhibited PA-induced lipid accumulation (Fig. 6B). Downregulation of PKC- β also reduced PA-induced apoptosis, oxidative stress, MTP collapse, and cytochrome *c* release (Fig. 6C and Supplementary Fig. S4D).

Further, RBX and ISV had an additive effect with PKC- β shRNA on inhibiting PA-induced apoptosis, ROS production, MTP collapse, and cytochrome *c* release. However, PKC- β

shRNA had no significant effect on p66Shc expression and mitochondrial translocation (Fig. 6D). ISV, but not RBX, had an additive effect with PKC- β shRNA on p66Shc expression (Fig. 6D). 12-Deoxyphorbol 13-phenylacetate 20-acetate (dPPA, 50 nM), a specific activator of PKC- β , promoted PA-induced apoptosis (Fig. 6E) and intracellular ROS production (Supplementary Fig. S2E) in rat hepatocytes. In addition, dPPA also impaired the protective effect of ISV against PA-induced apoptosis and oxidative stress in rat hepatocytes (Fig. 6E and Supplementary Fig. S2E). However, dPPA had no effect on ISV-mediated reduction of PA-induced lipid deposition in rat hepatocytes (Supplementary Fig. S2D). These results suggest that ISV ameliorated PA-induced apoptosis and lipid deposition through different mechanisms.

ISV inhibited PA-induced ER stress in rat hepatocytes

Changes in cellular redox state or abnormal accumulation of toxic lipid species can result in activation of compensatory response pathways, which comprise the unfolded protein response (UPR). The UPR stress signaling pathway is initiated by three main ER transmembrane proteins, protein kinase RNA-like endoplasmic reticulum kinase (PERK), inositol-requiring 1 α (IRE1 α), and activating transcription factor 6 (ATF6), which together promote transcription of genes designed to increase protein folding and degradation (22). As shown in Figure 7A–D, ISV significantly inhibited PA-induced upregulation of CHOP, XBP-1s, ATF4, IRE1 α , PERK, and ATF6 of both mRNA and protein levels in hepatocytes.

To confirm the effect of ISV on PA-induced hepatocellular ER stress, tauroursodeoxycholic acid (TUDCA), an inhibitor of ER stress, was administered. TUDCA inhibited PA-induced hepatocyte apoptosis (Fig. 7E), but it did not further enhance the protective effect of ISV, indicating that TUDCA and Isosteviol may share a similar mechanism against PA-induced damage by modulating ER stress response. TUDCA only had a slight effect on PA-induced ROS production and almost had no impact on the protective effect of ISV against PA-induced hepatocyte damage (Fig. 7F).

ISV protection of HFD-induced rat liver damage through PKC- β /p66Shc/ROS pathway and ER dysfunction

In vitro studies indicated that an ISV-mediated protective effect against PA-induced hepatic injury is not only mediated by inhibiting PKC- β expression and activity, but other PKC-

FIG. 5. Effect of p66Shc on PA-induced hepatic injury in rat primary hepatocytes. Rat primary hepatocytes were transfected with lentiviral p66Shc shRNAs for 48 h as described in the Materials and Methods section. (A) Representative immunoblots of p66Shc and β -actin are shown. Relative protein levels of p66Shc were determined based on the mean $\pm$ SD of three independent experiments, and actin was used as an internal loading control. Statistical significance compared with control group, ** p < 0.01, *** p < 0.001. (B, C) Rat primary hepatocytes were treated with 1 mM PA for 24 h after knocking down p66Shc. (B) The intracellular lipid, (C) cell apoptosis, ROS level, and MTP were measured by flow cytometry as previously described. Nile red fluorescence intensity, Annexin V positive cells ratio, high DCF intensity cells ratio, and JC-1 aggregates were calculated, respectively, based on the mean $\pm$ SEM of three independent experiments. (D, E) Rat primary hepatocytes were transfected with lentiviral p66Shc or p66Shc (S36D) phosphomimetic mutation for 24 h as described in the Materials and Methods section, and then they were treated with ISV (0, 1, 3, or 10 μ M) for 24 h. (D) Intracellular ROS levels were measured, and high DCF intensity cells ratio was calculated based on the mean $\pm$ SEM of three independent experiments. (E) The relative amount of LDH released to culture medium represents the mean $\pm$ SD of three independent experiments. * p < 0.05, *** p < 0.001, and NS, no significance. C-shRNA, control shRNA. Color images are available online.

β -independent mechanisms are also involved. To further identify the PKC- β -independent mechanisms underlying ISV-mediated protective effects on FFA/HFD-induced hepatic injury, the rats fed with HFD for 4 weeks were treated with RBX (40 μ g/kg/day), ISV (20 mg/kg/day), or control vehicle by oral gavage for another 4 weeks while on HFD.

In contrast to the regular diet group, the HFD-fed rats developed severe steatosis and lobular inflammation (Fig. 8A). Both RBX and ISV significantly reduced HFD-induced hepatic lipid deposition, hepatocyte injury, and dysregulation of serum lipids (Fig. 8A, B, and Supplementary Fig. S6A). However, compared with RBX, ISV showed much stronger effects on reducing HFD-induced liver injury (Fig. 8C), increasing SOD activity (Fig. 8D), and decreasing MDA levels (Fig. 8E), steatosis, and inflammation (Supplementary Fig. S6B, C). HFD induced PKC- β expression and increased phosphorylation of PKC- β in rat liver (Fig. 8F and Supplementary Fig. S6D); 20 mg/kg ISV and 40 μ g/kg RBX had no significant effect on PKC- β expression and phosphorylation.

Although ISV decreased phosphorylation of PKC- β slightly, there was no significant difference. ISV significantly inhibited HFD-induced p66Shc expression and phosphorylation. Although RBX had no significant effect on p66Shc expression in rat liver, RBX decreased phospho-p66Shc levels significantly (Fig. 8F and Supplementary Fig. S6D). ISV decreased phospho-p66Shc levels due to the inhibitive effect on PKC- β activity in rat liver. According to these data, Isosteviol protected rat hepatocytes against PA-induced injury through inhibiting PKC- β activity and p66Shc expression and phosphorylation.

Although the ER and mitochondria play distinct cellular roles, these organelles also form physical interactions with each other at sites defined as mitochondria-associated ER membranes (MAMs). MAMs play a key regulatory role in several cellular functions, including the transport of Ca^{2+} and lipids from ER to mitochondria (34). Several multifunctional sorting proteins, such as PACS-2 (phosphofurin acidic cluster sorting protein 2), have been identified as key players in controlling the ER-mitochondria communication and initiation of apoptosis (46). As shown in Figure 8G and Supplementary Figure S6E, HFD-induced upregulation of PACS-2 and IP3R1 (inositol 1,4,5-trisphosphate receptor type 1) was inhibited by ISV, but not by RBX, which decreased ER-mitochondria tethering or ER Ca^{2+} transport, subsequently reducing mitochondrial damage.

The electron microscope also showed that interaction between mitochondria and ER increased after HFD treatment, which was inhibited by ISV (Fig. 8H). Further, ISV significantly reduced HFD-induced activation of ER stress, as indicated by Western blot analysis of JNK and p38 activations and upregulation of the UPR genes, PERK, p-eIF2 α , GRP78, ATF4, and CHOP, but RBX had less effect (Fig. 8I and Supplementary Fig. S6F). Therefore, ISV reduced HFD-induced oxidative stress, increased SOD, and reduced MDA levels by modulating the PKC- β /p66Shc/ROS pathway in rat liver. Further, ISV also inhibited ER stress and protected against HFD-induced liver injury.

Discussion

NAFLD is the most common cause of chronic liver disease worldwide and is closely associated with cardiovascular disease, diabetes mellitus, and other metabolic diseases (44). The prevalence of NAFLD significantly increased during the past decade. The medical and societal cost of NAFLD became a major economic burden in the United States. The clinical spectrum of NAFLD ranges from NAFLD to non-alcoholic steatohepatitis (NASH), advanced fibrosis, cirrhosis, and, eventually, hepatocellular carcinoma (29).

The current understanding of the pathophysiology of NAFLD remains elusive. No effective pharmacological treatment is currently available to treat NAFLD other than diet control and managing physical activities. However, the adherence to lifestyle change is poor, especially for long-term weight loss diets, which also have harmful effects on the liver. Therefore, it is urgently needed to identify new therapeutic agents for the treatment and/or prevention of NAFLD progression.

Complementary and alternative medicine has been used in Asia for many years to treat various diseases, including NAFLD and other metabolic diseases. During the past decade, a number of potential candidates were tested in either preclinical studies or clinical trials for the treatment of NAFLD or the complications associated with the conventional therapies (5). However, most of the marketed herbal medicines are mixtures of different compounds. It is difficult to understand the functional mechanisms.

ISV is derived from stevioside, a widely used natural sweetener isolated from the leaves of *S. rebaudiana* Bertoni, by acid hydrolysis. Stevioside has been extensively used in a

FIG. 6. Effect of PKC- β on PA-induced hepatic injury in rat primary hepatocytes. Rat primary hepatocytes were transduced with lentiviral PKC- β shRNAs for 48 h as described in the Materials and Methods section. (A) Representative immunoblots of PKC- β and β -actin are shown. Relative protein levels of PKC- β were determined based on the mean $\pm$ SD of three independent experiments, and β -actin was used as an internal loading control. Statistical significance compared with control group, ** p < 0.01, *** p < 0.001. (B–D) Rat primary hepatocytes were further treated with PA (1 mM) with or without ISV (10 μ M) or RBX (20 nM) for 24 h. (B) The intracellular lipid, (C) cell apoptosis, ROS levels, and MTP were measured by flow cytometry as previously described. Nile red fluorescence intensity, Annexin V positive cells ratio, high DCF intensity cells ratio, and JC-1 aggregates were calculated, respectively, based on the mean $\pm$ SEM of three independent experiments. Statistical significance compared with control-shRNA or PA group, * p < 0.05, ** p < 0.01, and *** p < 0.001. (D) Representative immunoblot images of p66Shc in cytosol and mitochondria are shown. Cox4i1 and β -actin were used as internal loading controls for mitochondria and cytosol, respectively. Relative protein levels of p66Shc represent the mean $\pm$ SD of three independent experiments. Statistical significance compared with control-shRNA or PA group, * p < 0.05, ** p < 0.01. (E) Rat primary hepatocytes were treated with 50 nM dPPA with or without PA and ISV (0, 1, 3, and 10 μ M) for 24 h. Hepatocyte apoptosis was measured by flow cytometry. Annexin V positive cells ratio was calculated based on the mean $\pm$ SEM of three independent experiments. Statistical significance compared with PA group, * p < 0.05, ** p < 0.01, *** p < 0.001, and NS, no significance. dPPA, 12-deoxyphorbol 13-phenylacetate 20-acetate. Color images are available online.

variety of foods and products as a sugar substitute in the United States, Brazil, Japan, Korea, and China, but not in the Europe due to safety concerns. Although stevioside has been found to have multiple beneficial effects, its application is limited by its low bioavailability and the toxic and mutagenic effects of its metabolite, steviol (31). In contrast, studies have shown that ISV has therapeutic benefits against dyslipidemia, ischemia-reperfusion-induced organ injury, and IR, but no toxic and mutagenic effects (11). However, the functional mechanisms of ISV-mediated beneficial effects remain unclear. In this study, we identified that ISV prevents HFD/FFA-induced hepatic injury *via* modulating PKC- β /p66Shc/oxidative and ER stress pathways.

Mitochondrial dysfunction is associated with many diseases and pathological conditions, including NAFLD/NASH, diabetes, cardiovascular diseases, and other metabolic diseases, through the excessive production of ROS. Increasing amounts of evidence indicate that control of mitochondrial function and reduction of ROS production are beneficial in many oxidative stress-associated diseases (37).

p66Shc is a redox enzyme that mediates H₂O₂ formation *via* the oxidation of cytochrome *c* and plays a critical role in mitochondrial ROS production. It has been reported that p66Shc-deficient mice are protected from many diseases caused by oxidative stress (15), suggesting that the specific inhibitor of p66Shc has promising therapeutic potential. It also has been reported that p66Shc-mediated signaling contributes to the disease progression of NAFLD (18a). However, due to the lack of a p66Shc inhibitor, a significant amount of effort has been made to modulate the upstream pathways of p66Shc.

It has been identified that PKC- β is an important regulator of p66Shc activity. PKC- β -mediated phosphorylation of p66Shc on serine 36 is critical to Pin1 binding and mitochondrial translocation (12, 15). PKC- β deficient mice exhibit resistance to HFD-induced obesity and IR and a marked reduction in the whole body TG content, including white adipose tissue mass (16). Although interfering PKC- β activity holds potential for the development of novel therapeutic approaches for the prevention of oxidative stress-associated metabolic diseases, such as NAFLD/NASH, none of the available PKC- β inhibitors has been approved for clinical use to treat NAFLD/NASH.

A hallmark of PKC- β activation is the translocation of its targets between intracellular compartments. PKC- β -mediated phosphorylation of p66Shc induces its transloca-

tion into mitochondria and results in ROS production (13, 24). It has been reported that p66Shc expression is increased in HFD-induced NAFLD rodent models and high p66Shc activity contributes to HFD/FFA-induced lipotoxicity (18a, 35). However, whether there is a change of p66Shc mitochondrial translocation remains unclear. Here, we found that ISV not only inhibited PA-induced p66Shc expression but also inhibited PA-induced p66Shc mitochondrial translocation through inhibition of PKC- β activity, which resulted in a decrease of intracellular ROS generation (Fig. 4).

Kelmer Bracht *et al.* (18) had reported that ISV inhibited oxidative phosphorylation, ATPase activity, NADH-oxidase activity, succinate-oxidase activity, succinate dehydrogenase, and L-glutamate dehydrogenase. ISV also inhibited succinate-induced net proton ejection and several substrates-induced mitochondria swelling. A previous study reported that ISV dose dependently inhibited mitochondria swelling from 0.05 to 1.0 mM (7). At 1 mM concentration, ISV inhibited KNO₃ and valinomycin-induced rat mitochondria swelling by 47%. The maximum concentration used in this study is 0.01 mM, which is unlikely to impact mitochondrial function.

RBX, a PKC- β specific inhibitor, has been approved to reduce the burden of microvascular complications for patients with diabetes (1, 17) and to delay the development of diabetic kidney disease (45). We found that RBX also protected rat primary hepatocytes from PA-induced hepatocyte apoptosis (Fig. 4) and HFD-induced liver damage in rats (Fig. 8). In addition to targeting PKC- β , PP2A, which dephosphorylates p66Shc after isomerization by Pin1, also plays an important role in p66Shc mitochondrial translocation (33). Kwan *et al.* reported that PA increased PP2A expression in HepG2 cells (21). However, in our study, PA had no effect on PP2A expression in primary hepatocytes (Supplementary Fig. S3). In addition, ISV had no effect on the expression of PP2A and Pin1 as well as on PP2A activity (Supplementary Fig. S3).

Therefore, a decrease in p66Shc mitochondrial translocation is mainly due to the inhibition of PKC- β expression and activity by ISV (Fig. 4 and Supplementary Fig. S3). Although RBX is more potent than ISV in inhibiting PKC- β expression and activity, it has less protective effect on PA-induced hepatic lipid accumulation and apoptosis in hepatocytes (Fig. 4) and HFD-induced liver injury (Fig. 8). This suggests that other PKC- β -independent mechanisms are also involved in ISV-mediated protective effects on FFA/HFD-induced hepatic injury.

FIG. 7. ISV protected PA-induced hepatocyte damage through inhibition of ER stress. Rat primary hepatocytes were treated with PA (1 mM) with or without different concentrations of ISV (0, 1, 3, and 10 μ M) for 24 h. (A, B) The mRNA levels of CHOP, XBP1s, ATF4, IRE1 α , PERK, and ATF6 were measured by real-time RT-PCR and normalized by using GAPDH as an internal control as described in the Materials and Methods section. The data represent the mean $\pm$ SD of three independent experiments. Statistical significance compared with control or PA group, * p < 0.05, ** p < 0.01, and *** p < 0.001. (C, D) The protein levels of CHOP, XBP1s, ATF4, IRE1 α , PERK, and ATF6 were determined by Western blot analysis. Lamin B1 and β -actin were used as loading controls for nuclear protein and cytosolic protein, respectively. The representative images of immune blots are shown. Relative protein levels represent the mean $\pm$ SD of three independent experiments. Statistical significance compared with control or PA group, * p < 0.05, ** p < 0.01. (E, F) Rat primary hepatocytes were treated with TUDCA with or without PA (1 mM) and ISV (0, 1, 3, and 10 μ M) for 24 h. (E) Hepatocyte apoptosis and (F) ROS production was measured by flow cytometry. Annexin V positive cells ratio and high DCF intensity cells ratio were calculated, respectively, based on the mean $\pm$ SEM of three independent experiments. Statistical significance compared with PA group, ** p < 0.01, *** p < 0.001, and NS, no significance. ATF, activating transcription factor; ER, endoplasmic reticulum; IRE1 α , inositol-requiring 1 α ; PERK, protein kinase RNA-like endoplasmic reticulum kinase; RT-PCR, reverse transcription-polymerase chain reaction; TUDCA, tauroursodeoxycholic acid.

Berniakovich *et al.* reported that insulin activates the redox enzyme activity of p66Shc specifically in adipocytes and that p66Shc-generated ROS regulate insulin signaling through multiple mechanisms, including AKT phosphorylation, Foxo localization, and regulation of selected insulin target genes

(6). Therefore, p66Shc signal-related oxidative stress might promote fat deposition and fat-related disorders. p66Shc signal might have similar effects on lipid accumulation in hepatocytes. HFD-induced NAFLD and PA-induced hepatocyte damage accompany enhanced p66Shc activity and

lipid accumulation. Although ISV inhibited p66Shc translocation and the following oxidative stress in hepatocytes, which might be beneficial to a decrease in intracellular lipid deposition, there are also other mechanisms for the decrease in intracellular lipid accumulation after ISV treatment. ER stress attenuation and genes related to lipid synthesis and lipolysis changes might be affected by ISV treatment.

In addition to oxidative stress, ER stress is also a major contributor to metabolic diseases, including NAFLD/NASH (3). The mitochondrion is interconnected with the ER. The junction area between the mitochondrion and ER is involved in the regulation of lipid synthesis, Ca^{2+} signaling, mitochondrial biogenesis, and intracellular trafficking (34).

It has been reported that tunicamycin-induced acute ER stress *in vivo* results in an increase of ER-mitochondria interaction, oxidative stress, and impaired metabolic homeostasis. However, downregulation of PACS-2 or IP3R1, key proteins for ER-mitochondria tethering or Ca^{2+} transport, respectively, improves mitochondrial oxidative capacity and glucose metabolism in obese animals (2). Our results showed that ISV, but not RBX, inhibited PA-induced increases of PACS-2 and IP3R1 expression in rat primary hepatocytes (Fig. 8G). Consequently, ISV may decrease ER-mitochondria interaction and inhibit PA-induced hepatic ER stress.

In summary, our results suggest that ISV prevents FFA-/HFD-induced hepatic lipotoxicity *via* inhibiting PKC- β activity, p66Shc expression and mitochondrial translocation, ROS production, and ER stress and modulating ER-mitochondria interaction. Although targeting PKC- β has a beneficial effect, ISV has more profound effects than RBX because it targets multiple pathways (Fig. 9). In conclusion, ISV represents a promising therapeutic agent for NAFLD.

Materials and Methods

Materials

ISV was produced by the acidic hydrolysis of stevioside (purchased from Stevioside Sweetener Factory of Nankai University, Tianjin, China) according to procedures previously described in the literature (4). Ruboxistaurin (SML0693), Cantharidin (C7632), Nile red (72485), 2',7'-dichlorofluorescein diacetate (D6883), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (M2128) were purchased from

Sigma-Aldrich (St. Louis, MO); dPPA (sc-287296) was purchased from Santa Cruz Biotechnology (Dallas, TX). Lipofectamine 2000 (11668030), MitoTracker Green FM (M7514), and JC-1 (T3168) were purchased from Thermo Fisher (Rockford, IL). Cytotoxicity Detection Kit Plus (LDH) (04 744 926 001) was purchased from Roche Applied Science (Mannheim, Germany). Caspase-3 Assay Kit (ab39401) was purchased from Abcam (Cambridge, MA). PPase-2A Catalytic Subunit (V6311), Serine/Threonine Phosphatase Assay System (V2460), and ADP-Glo™ Kinase Assay and PKC- β II Kinase Enzyme System (V9701) were purchased from Promega (Madison, WI).

Antibodies against β -actin (sc-7210), Lamin B1 (sc-377000), p53 (sc-71820), and CHOP (sc-71136) were purchased from Santa Cruz Biotechnology (Dallas, TX). Antibodies against Cytochrome *c* (556432) and GRP78 (610979) were purchased from BD Biosciences (San Jose, CA). Antibodies against phospho-PKC- β (9371), Pin1 (3722), XBP1s (12782), IP3R1 (8568), JNK (9252), phospho-JNK (9255), p38 (9212), phospho-P38 (9216), PERK (3192), phospho-PERK (3179), phospho-eIF2 α (9721), eIF2 α (2103), anti-mouse IgG, horseradish peroxidase (HRP)-linked antibody (7076), and anti-rabbit IgG, HRP-linked antibody (7074) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against p66Shc (ab131515), phospho-p66Shc (phospho S36) (ab54518), PKC- β (ab32026), PP2A (ab106262), IRE1 α (ab37073), ATF6 (ab203119), and ATF4 (ab1371) were purchased from Abcam. Antibodies against PACS-2 (PA5-43701) and cytochrome *c* oxidase subunit 4i1 (Cox4i1) (MA5-15686) were purchased from Thermo Fisher (Rockford, IL).

The chips of human liver tissues were purchased from Alenabio Biotechnology Co., Ltd. Using these chips for science research was approved by the Ethics Committees of Tongxu People Hospital.

Animals

Male Sprague Dawley rats (200 ± 20 g) were purchased from Experimental Animal Center of Nanjing Medical University (Nanjing, China). The rats were housed under 12 h light/12 h dark cycle and fed standard chow and tap water *ad libitum* for a week before treatment. The rats were divided into two groups, ND group and HFD group, and fed either a regular chow diet or HFD (containing 88% ordinary forage, 10% lard, and 2% cholesterol [w/w]), respectively. After 4 weeks, the HFD group was further randomly divided into

FIG. 8. Effect of ISV and RBX on HFD-induced liver injury in rats. Rats were fed with regular rodent diet or HFD for 4 weeks and then, the HFD-fed group rats were treated with ISV (20 mg/kg) or RBX (40 $\mu\text{g/kg}$) by oral gavage daily for 4 weeks. (A) Representative images of H&E staining of the liver tissues from each treatment group are shown. $N=6-8$ rats per group. (B) The serum lipid levels. (C) The enzyme activities of ALT and AST in the serum. (D) Hepatic SOD activity normalized with total protein amount. (E) Hepatic MDA levels normalized with total protein amount. The values are presented as mean $\pm$ SD. $N=6-8$ rats per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and NS, no significance. (F) Representative immunoblot images of p-PKC- β , PKC- β , p-p66Shc, p66Shc, and β -actin are shown. (G) Representative immunoblot images of PACS-2, IP3R1, and β -actin are shown. (H) Representative images of electron microscopy showing mitochondria-associated ER membrane. The white arrows present the increased ER-mitochondria interaction. (I) Representative immunoblot images of cytosolic stress-responsive genes and nuclear ER stress genes, including p-JNK, JNK, p-p38, p38, p-PERK, PERK, p-eIF2 α , GRP78, ATF4, and CHOP. Actin and Lamin B were used as loading controls for cytosolic and nuclear proteins, respectively. IP3R1, inositol 1,4,5-trisphosphate receptor type 1; JNK, c-Jun N-terminal kinase; PACS-2, phosphofurin acidic cluster sorting protein 2. Color images are available online.

FIG. 9. Schematic diagram of the potential signaling pathways involved in ISV-mediated protective effect on HFD/FFAs-induced hepatic lipotoxic injury. HFD feeding increases FFAs in circulation, which upregulates p66Shc expression and activation of PKC- β . Activated PKC- β phosphorylates p66Shc, allowing its recognition by Pin1 and dephosphorylation by PP2A and the transfer from the cytosol to the mitochondrion, where p66Shc induces free O_2^- production and ROS and results in cytochrome *c* release. ROS can further activate PKC- β . In the meantime, FFAs also induces ER calcium depletion by upregulating IP3R1/PACS-2 and activates the UPR. Increased cytosolic cytochrome *c* and ROS and upregulation of ATF4/CHOP promote cell apoptosis. ISV not only inhibits ROS and ER stress but also inhibits PKC- β activation, which prevents mitochondrial translocation of p66Shc and inhibits ROS production. In addition, ISV also inhibits p66Shc expression. Cyto-*c*, cytochrome *c*; Pin1, peptidyl-prolyl cis-trans isomerase NIMA-interacting 1; PP2A, serine/threonine protein phosphatase 2A; TOM, translocase of the outer membrane; UPR, unfolded protein response. Color images are available online.

vehicle control group and treatment groups ($n=6-8$). In the first set of *in vivo* experiments, the four groups were ND, HFD, HFD+ISV (10 mg/kg), and HFD+ISV (20 mg/kg). Our previous study showed that 5 mg/kg ISV had only marginal effect on high-fat emulsion-induced TG increase (51).

To further examine the protective effect of ISV on NAFLD, we used HFD that contained moderate lard and cholesterol to induce the rat NAFLD model and increased the dosage of ISV in this study. In the second experiment, the four groups were ND, HFD, HFD+RBX (40 μ g/kg), and HFD+ISV (20 mg/kg). Animals were gavaged daily for 4

weeks. Rats were weighed once a week to adjust the drug intake. At the end of the experiment, all rats were sacrificed after 12 h of fasting. Blood samples and liver tissues were collected. All studies were approved by the Animal Study Committee of Southeast University (Nanjing, China) and done in accordance with the Guide for the Care and Use of Laboratory Animals.

Blood chemistry

Serum TC, TG, LDL-cholesterol, high-density lipoprotein (HDL)-cholesterol, ALT, and AST were detected with a biochemistry automatic analyzer (Beckman Coulter, Indianapolis IN). FFAs, MDA, and SOD in liver homogenates were measured by using colorimetric detection kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). FFAs were converted to their coenzyme A derivatives, which were subsequently oxidized, leading to formation of color and can be measured at 540–550 nm. MDA was measured with the TBA method, and the activity of SOD was measured by the WST-1 method.

Histopathology analysis

The hematoxylin- and eosin-stained samples were examined blindly by a professional pathologist to evaluate the presence of ballooning, steatosis, inflammation, and fibrosis based on a 4-point scale: For steatosis: grade 0, absent; grade 1, <33% of the parenchyma; grade 2, 34%–66% of the parenchyma; and grade 3, >67% of the parenchyma. For inflammation: grade 0, absent of foci of inflammation; grade 1, fewer than one foci per two 20 $\times$ fields; grade 2, one foci per two 20 $\times$ fields; grade 3, one to two foci per one 20 $\times$ field; or grade 4, more than two foci per one 20 $\times$ field (8, 47).

Isolation and culture of primary rat hepatocytes

Primary hepatocytes were isolated from male Sprague Dawley rats (250–300 g) and cultured as described (36, 49). An adult rat was anesthetized with intraperitoneal 87 mg/kg ketamine +13 mg/kg xylazine. An 18-gauge angiocath was inserted into the hepatic portal vein. The perfusate tubing was connected to the needle and the infusion was initiated *in situ* at a low flow to 10 mL/min with prewarmed (37 $^{\circ}$ C) perfusion buffer I: 0.9 mM $MgCl_2$, 0.5 mM EDTA, and 25 mM HEPES in HBSS (without Ca^{2+} , and Mg^{2+}). Once successful cannulation was confirmed, a cut was made at the inferior vena cava to allow efflux. The rate of flow was increased to 25 mL/min. The perfusion solution was switched to perfusion buffer II: 25 mM HEPES and 3.3 U/mL collagenase II in HBSS (without Ca^{2+} , and Mg^{2+}) for an additional 6 min.

The liver was dissected and the cells were dispersed into William's complete medium. The cell dispersion was filtered through a 100- μ m pore size cell strainer to remove connective tissues and undigested tissue fragments. The cells were suspended in 40 mL William's complete medium and centrifuged at 50 *g* for 3 min at 4 $^{\circ}$ C. The supernatant was aspirated, and the cells were gently resuspended in 40-mL cold William's complete medium to wash the cells. Centrifugation was repeated, and the cells were diluted with warm William's complete medium to a preferred concentration.

Apoptosis analysis

Rat primary hepatocytes were treated with 1 mM PA with or without various concentrations of ISV for 24 h. Cells were collected, washed with cold phosphate-buffered saline, then resuspended in 400 μ L of reaction solution (10 mM of 4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid, 140 mM of NaCl, and 2.5 mM of CaCl_2 ; pH 7.4), and finally stained with Annexin V-FITC and PI. The apoptotic cells were detected with a BD FACSCalibur flow cytometer.

Mitochondrial transmembrane potential

Rat primary hepatocytes or HepG2 cells were treated with 1 mM PA with or without various concentrations of ISV for 24 h. The MTP was measured by using the fluorescent probe JC-1 and a BD FACSCalibur flow cytometer, as previously described (49).

Caspase-3 activity assay

Rat primary hepatocytes or HepG2 cells were treated with 1 mM PA with or without various concentrations of ISV for 24 h. Caspase-3 activity in the hepatocyte lysate was measured by a colorimetric assay kit according to the manufacturer's instructions (38).

Determination of cytotoxicity

Rat primary hepatocytes were treated with 1 mM PA with or without various concentrations of ISV for 24 h. Cytotoxicity was determined by measuring the release of LDH into the medium according to the manufacturer's instructions for the cytotoxicity detection kit (Roche Applied Science, Indianapolis, IN).

Measurement of intracellular ROS

Rat primary hepatocytes or HepG2 cells were treated with 1 mM PA with or without various concentrations of ISV for 24 h. The cells were collected and incubated with 20- μ M 2,7-dichlorofluorescein diacetate at 37°C for 30 min. Intracellular ROS levels were measured by a BD FACSCalibur flow cytometer and expressed by DCF fluorescence intensity (49).

Western blot analysis

The total cell lysate, mitochondrial lysate, and nuclear lysate were prepared and processed as described (48). The protein expression levels of cytochrome *c*, p66Shc, Pin1, PP2A, PACS-2, and IP3R1 were detected with specific antibodies. β -Actin, Cox4i1, and Lamin B1 were used as loading controls for cytosolic, mitochondrial, and nuclear proteins, respectively.

PKC- β activity assay

The PKC- β activity was detected by using the ADP-Glo Kinase Assay and PKC- β II Kinase Enzyme System from Promega in accordance with the manufacturer's instructions.

PP2A activity assay

The PP2A activity was measured by using an Ser/Thr PPase Assay kit from Promega according to the manufacturer's instructions.

RNA isolation and real-time reverse transcription-polymerase chain reaction analysis

Total RNA from rat primary hepatocytes was extracted and isolated by using TRIzol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. The first strand of cDNA was reverse transcribed by using the ThermoScript RT-PCR system kit (Life Technologies) at 50°C for 50 min. The mRNA levels of targeted genes were determined by real-time-polymerase chain reaction (PCR) as previously described (53, 54).

The following primers were used for GAPDH: forward 5'-AGGTCGGTGTGAACGGATTG-3', reverse 5'-TGAGACCATGTAGTTGAGGTCA-3'; PKC- β : forward 5'-AGCGAGACACC TCCAACCTTC-3', reverse 5'-CAG TGGGAGTCAGTTCCACAG-3'; p66Shc: forward 5'-AAGTACAACCCACTTCGGAATG-3', reverse 5'-GAAAGAAGGAACACAGGGTAGTC-3'; IRE1 α : forward 5'-TGGACGGACA GAATACACCA-3', reverse 5'-TGGACACAAAGTGGGACATC-3'; CHOP: forward 5'-CTGGA AGCCTGGTATGAGGAT-3'; reverse 5'-CAGG GTCAAGAGTAGTGAAGGT-3'; ATF4: forward 5'-ATGGC GCTCTTCACGAAATC-3', reverse 5'-ACT GGTCGAAGGG GTCATCAA-3'; PERK: forward 5'-TAATCCATTCTCCG TCTA-3', reverse 5'-GATAGTAACCGTTGTCATA-3'; ATF6: forward 5'-TAATGGTGCTAAGTGAAGA-3', reverse 5'-AC TCTGTCGTGTTAATGA-3'; XBP1u: forward 5'-CAGAGTC CAAGGGGAATGG-3', reverse 5'-CTGCAGAGGCGCACG TAGT-3'; XBP1s: forward 5'-AGTCCGCAGCAGGTGCA GGCC-3', reverse 5'-GGGTCCAACCTT GTCCAGAATG-3'. p53: forward 5'-ACCGTATGAGCCACCTGA-3', reverse 5'-GGAGTCTTCCAGCGTGATG-3'.

RNA interference with shRNA and transduction of rat primary hepatocytes

The shRNA sequences for PKC- β RNA interference are PKC- β shRNA #1: GCGACACCTGTATGATGAATGT CCA and PKC- β shRNA #2: ACGAAACCTTCA GATTT-CAGCTGAA; the shRNA sequences for p66Shc are p66Shc shRNA #1: GCAACTTGAAGCTGGCCAATC and p66Shc shRNA2: GCTGCATCCCAACGACAAAGT; and the sequence for the control is no target shRNA: CAACAA-GATGAAGAGCACCAA. According to the manual (26), pLKO.1-TRC cloning vector was used to construct the shRNA plasmid. The recombinant construct was co-transfected together with two packaging vectors psPAX2 and pMD2.G into 293FT cells. Purified lentiviral particles were used to transduce rat primary hepatocytes.

Statistical analysis

Flow cytometry results are expressed as the mean $\pm$ standard error of the mean; other results are expressed as the mean $\pm$ standard deviation. Significance between groups was evaluated by one-way analysis of variance followed by a Student-Newman-Keuls *post hoc* test. Statistics were performed by using Sigma Plot. $p < 0.05$ was considered statistically significant.

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Authors' Contributions

H.Y., X.W., and H.Z. conceived the original ideas, designed the study, analyzed the data, and wrote the article; H.Y., D.X., X.W., F.X., and L.L. carried out the experiments and data analysis.

Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

ALT = alanine aminotransferase
AST = aspartate aminotransferase
ATF6 = activating transcription factor 6
Cox4i1 = cytochrome *c* oxidase subunit 4i1
DCF = 2',7'-dichlorofluorescein
dPPA = 12-deoxyphorbol 13-phenylacetate 20-acetate
ER = endoplasmic reticulum
FFA = free fatty acid
FITC = fluorescein isothiocyanate

HDL = high-density lipoprotein
HFD = high fat diet
HRP = horseradish peroxidase
IP3R1 = inositol 1,4,5-trisphosphate receptor type 1
IR = insulin resistance
IRE1 α = inositol-requiring 1 α
ISV = Isosteviol
JC-1 = 5,5,6,6-tetrachloro-1,1,3,3-tetraethyl benzimidazolylcarbocyanine iodide
JNK = c-Jun N-terminal kinase
LDH = lactate dehydrogenase
LDL = low-density lipoprotein
MAMs = mitochondria-associated ER membranes
MDA = malondialdehyde
MTP = mitochondrial transmembrane potential
NAFLD = nonalcoholic fatty liver disease
NASH = nonalcoholic steatohepatitis
ND = normal diet
p66Shc = Src-homology-2-domain-containing transforming protein 1
PA = palmitic acid
PACS-2 = phosphofurin acidic cluster sorting protein 2
PERK = protein kinase RNA-like endoplasmic reticulum kinase
PI = propidium iodide
Pin1 = peptidyl-prolyl cis-trans isomerase
NIMA-interacting 1
PKC- β = protein kinase C- β
PP2A = serine/threonine protein phosphatase 2A
RBX = Ruboxistaurin
ROS = reactive oxygen species
RT-PCR = reverse transcription-polymerase chain reaction
SD = standard deviation
SEM = standard error of the mean
SOD = superoxide dismutase
TC = total cholesterol
TG = triglyceride
TUDCA = tauroursodeoxycholic acid
UPR = unfolded protein response