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Shc inhibitor idebenone ameliorates liver injury and fibrosis in dietary NASH in mice

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Abstract

Shc expression rises in human nonalcoholic steatohepatitis (NASH) livers, and Shc-deficient mice are protected from NASH–thus Shc inhibition could be a novel therapeutic strategy for NASH. Idebenone was recently identified as the first small-molecule Shc inhibitor drug. We tested idebenone in the fibrotic methionine-choline deficient (MCD) diet and the metabolic fast food diet (FFD) mouse models of NASH. In the fibrotic MCD NASH model, idebenone reduced Shc expression and phosphorylation in peripheral blood mononuclear cells and Shc expression in the liver; decreased serum alanine aminotransferase and aspartate aminotransferase; and attenuated liver fibrosis as observed by quantitative polymerase chain reaction (qPCR) and hydroxyproline quantification. In the metabolic FFD model, idebenone administration improved insulin resistance, and reduced inflammation and fibrosis shown with qPCR, hydroxyproline measurement, and histology. Thus, idebenone ameliorates NASH in two mouse models. As an approved drug with a benign safety profile, Idebenone could be a reasonable human NASH therapy.

Keywords

fibrosis; idebenone; inflammation; NASH; Shc

1 | INTRODUCTION

Multiple pathophysiologies including insulin resistance, steatosis, inflammation, and progressive fibrosis underlie nonalcoholic fatty liver disease/steatohepatitis (NAFLD/NASH). The global prevalence of NASH is on the rise.[1] NASH is soon to be the leading cause of liver transplantation in the United States.[2] With no approved treatment, there is an urgent need to discover effective therapeutic targets and treatments.

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CONFLICT OF INTERESTS

The authors have no conflict of interest to disclose.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Shc and IRS are both substrates of insulin receptor tyrosine kinase and compete for the docking site on the NPE motif ^{960}Y. Therefore, Shc competitively inhibits IRS phosphorylation and its downstream activation of the phosphoinositide 3-kinase (PI3K)/Akt pathway.[3–6] Under conditions of rodent insulin resistance, the Shc pathway becomes activated.[7,8] Shc also rises in human type 2 diabetes.[9,10] Shc has been demonstrated to increase in human and mouse NASH as well.[11,12] And conversely, genetic reduction of Shc is protective in dietary type 2 diabetes, NASH, and liver fibrosis.[12–15]

Idebenone was recently identified as the first Shc inhibitor, by a high throughput screen of 1600 clinically approved drugs.[16] Idebenone's binding of the p52Shc protein blocks the access of p52Shc to the insulin receptor's phosphotyrosines. In addition to being insulin-sensitizing through increasing the IRS1-dependent Akt response, idebenone was also demonstrated to be hepatoprotective.[16,17] As idebenone has been available to experimenters since the late 1980s, it has been used by multiple experimenters and observed to be hepatoprotective in multiple models of liver injury. Idebenone was found protective in sodium nitrateinduced liver hypoxia.[18] Idebenone protected from fibrotic consequences of titanium dioxide exposure.[19,20] Tiefenbach showed that very high doses of idebenone improved hepatosteatosis in db/db mice.[17] Although most of these authors attributed idebenone's hepatoprotection to its antioxidant properties that were only observed at high concentrations greater than 1000 nM, we attribute idebenone's hepatoprotective properties to its ability to engage and inhibit the Shc protein, which occurs at an IC50 of ~100 nM, and its cytoprotective potency which occurs at an IC50 of ~100 to 300 nM depending on cell type.[16] Idebenone is directly cytoprotective to hepatocytes against palmitate toxicity.[16]

Given that idebenone was recently found to be a much more potent Shc inhibitor than antioxidant, is approved for human use in Europe, and as both Shc inhibition and idebenone administration have been shown to be hepatoprotective in animal models of liver hypoxia, fibrosis, and steatosis, the aim of this study is to test idebenone in two mouse models of dietary NASH, the methionine-choline deficient diet (MCD) model in which substantial fibrosis occurs, and the fast food diet (FFD) model which has the metabolic and fibrotic sequelae of NASH, and we have observed significant benefits in both models. Our hypothesis for idebenone's hepatoprotective mechanism is summarized in Figure 1.

2 | MATERIALS AND METHODS

2.1 | Animals, diet, and dosing

All the animal experiments were approved by Institutional Animal Care and Use Committee at UC Davis. Male C57B6/J mice were housed in a pathogen-free facility with 12:12 h light/dark cycles and ad libitum access to food and water. Standard polycarbonate cages with wire-top lids, autoclavable soft paper bedding with paper-chip were used. The mice were singly housed during the experiments and were randomly assigned to diet and drug treatments by body weights. Animals were monitored daily by vivarium staff. For the MCD model, the 8-week old mice were given either a control diet supplemented with methionine and choline or an MCD diet (Bio-Serv) for 6 weeks. Idebenone (MilliporeSigma) was formulated in peanut butter and dosed to the mice at 10 or 40 mg/kg in the same volume (100 μl) in a voluntary manner from Day 1 of the diet. The dosing process was described

previously.[21] In brief, the mice were given 100 μl plain peanut butter for 3–5 days before the drug dosing. The mice normally finished the pellet within few minutes after the training. All the animals included were compliant with the dosing. The same amount of peanut butter as the vehicle was given to the control group and one MCD group. There were 6–8 mice for each experimental group. For the FFD model, aged mice (10–12 months) were used as they develop more significant fibrosis.[12] The mice were placed on control chow or FFD (Bio-Serv, AIN-76A), supplemented with glucose 18.3 g/L and fructose 23.1 g/L in drinking water, for 16 weeks. Idebenone 20 mg/kg oral daily was started at 9th week for 8 weeks long till the endpoint. The Chow group and one FFD group were given the same amount of peanut butter. There were 6–8 mice for each group.

The mice were euthanized at the endpoint for liver tissue and serum collection.

2.2 | Glucose tolerance test (GTT) and insulin tolerance test (ITT)

The mice on FFD were subjected to GTT and ITT at the 15th week of the diet following the standard procedures approved by IACUC. In brief, for GTT, the mice were fasting for 16 h, with access to freshwater supply, followed by an i.p. injection of glucose/saline solution (2 g/kg). Blood was collected from tail nick and glucose levels were recorded at the time points indicated. For ITT, after 4 h of fasting, the mice were i.p. injected with insulin 1 U/kg, and blood glucose was measured at the time points as shown. The area under the curve (AUC) for GTT and ITT was calculated following the standard trapezoidal rule.

MCD diet is known to induce significant histologic changes in the liver simulating human NASH, although it does not cause insulin resistance. Therefore, these mice were analyzed for liver histology and fibrosis to evaluate the effect of idebenone, while GTT and ITT were not conducted.

2.3 | Peripheral blood mononuclear cell (PBMC) isolation

Five microliters of blood were collected from the tail vein and incubated with red blood cell lysis buffer (Abcam) for 10 min, after washing with phosphate-buffered saline for two times, and brief centrifugation, the PBMC enriched cell pellets were lysed in a lysis buffer (MilliporeSigma) containing protease inhibitors and phosphatase inhibitors (MilliporeSigma) and subjected to Jess protein analysis.

2.4 | Conventional Western blot and automated Western blot (Jess capillary Western blot) analysis

Liver tissues were homogenized in the lysis buffer (MilliporeSigma) containing protease and phosphatase inhibitors (MilliporeSigma). Thirty micrograms of proteins were separated proteins subjected to 4%–20% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. After blocking, the membranes were probed with primary antibodies as indicated at 4°C overnight, then with fluorescence conjugated secondary antibodies (LI-COR) for 1 h at room temperature. Images were visualized and analyzed using Odyssey CLx Imaging System (LI-COR).

To detect phospho-Shc and Shc in PBMC, automated Western blot analysis was conducted using the Jess™ capillary western system (ProteinSimple) following the instructions. Total 0.75 μg of protein was separated and immobilized in electrophoretic capillaries provided by the manufacturer. Primary antibodies (1:50 to 1:100) were applied for 1 h. After probing with appropriate secondary antibodies for another hour, the signal was acquired and quantified with Compass software (ProteinSimple).

Anti-phospho-Shc (Tyr239/240), and anti-Shc were from Cell Signaling Inc.; anti-β-Actin was from R&D Systems.

2.5 | Serologic studies and liver histology

Mouse serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) measurement were conducted by the Comparative Pathology Laboratory at UC Davis. Liver tissue hematoxylin and eosin (H&E) staining and picrosirius red were performed by the Department of Pathology, School of Veterinary Medicine, UC Davis.

2.6 | RNA extraction and real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

RNA was extracted from liver tissues using the QIAGEN RNeasy Mini Kit following the instructions. cDNA was synthesized with an iScript cDNA synthesis kit (Bio-Rad). Absolute real-time PCR was conducted with the SYBR Green DNA Master mix (Applied Biosystems) using the Applied Biosystems ViiA 7 real-time PCR system (Thermo Fisher Scientific). The absolute amount of target genes was calculated with a standard curve generated from a series amounts of templates. The result was normalized to the housekeeping gene Arbp. Primers are listed in Table 1.

2.7 | Hydroxyproline assay

Collagen in liver tissue was quantified with hydroxyproline assay using a colorimetric kit from Abcam following the instructions. The result was obtained based on a standard curve generated from a serial dilution of the hydroxyproline standard and expressed as milligrams of hydroxyproline per gram of wet liver.

2.8 | Statistical analysis

The data are expressed as the mean± *SD*. Differences between two groups were compared using a one-way analysis of variance (ANOVA) associated with Dunnett's test. The 2-tailed, unpaired Student *t* test was used to analyze the differences between the two groups. Comparisons between more than two groups were done with Kruskal–Wallis test, followed by Dunn's multiple comparison test. Statistical significance was considered when $p < 0.05$.

3 | RESULTS

Idebenone did not cause significant adverse effects on any dosing groups in either MCD or FFD model. Idebenone did not alter the changes of body weight or liver weight/body weight ratio caused by the NASH diets (Figure S1).

3.1 | Idebenone inhibits p52Shc phosphorylation and expression in PBMC and decreases Shc expression in the liver in MCD diet-induced liver injury

Mice were fed with an MCD diet for 6 weeks. Idebenone 10 or 40 mg/kg was dosed from Day 1 through the end. To confirm the in vivo target engagement, PBMCs were collected for capillary Western blot analysis (Figure 2A) to analyze p52Sch expression and activation. Shc expression and phosphorylation increased in PBMC in MCD mice and these were suppressed by idebenone on both dosages. In the liver, with idebenone treatment, a trend of decrease of Shc expression was shown (Figure 2B).

3.2 | Preventive application of idebenone ameliorates liver injury and fibrosis in MCD diet-fed mice in a dose-dependent manner

Serum analysis from the above mice showed that ALT level (Figure 3A) was significantly reduced at 40 mg/kg, while AST (Figure 3B) had a trend of decrease at both 10 and 40 mg/kg groups. The liver tissues were subjected to real-time quantitive PCR (qPCR) to assess transcripts of profibrogenic markers collagen 1a1, α-smooth muscle actin (αSMA), transforming growth factor-β (TGFβ), matrix metalloproteinase 2 (MMP2), and Timp1 (Figure 3C). Col1a1 was reduced by idebenone in a dose-dependent manner; all the other genes were reduced at both dosage groups significantly ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). This was confirmed by hydroxyproline measurement (Figure 3D) and picrosirius red staining (Figure 3E). Liver histology showed fewer infiltrating flammatory cells in both treated groups (Figure 3E).

3.3 | Idebenone reduces p52Shc phosphorylation in PBMC in FFD fed mic

We next examined the therapeutic effect of idebenone in the FFD model, which has been considered close to human NASH with significant insulin resistance, steatohepatitis, and fibrosis.[22] Aged mice (above 10 months) were given FFD for 16 weeks. To test the therapeutic effect of the drug on established disease, idebenone (20 mg/kg) was started at week 9 through the end. The aged mice were used as they tend to develop more severe liver injury and fibrosis.[12] PBMC from FFD mice showed higher p52Shc phosphorylation and it was blunted in the idebenone group (Figure 4A). The expression of Shc increased as well ($^{*}p < 0.05$), but no significant reduction was observed in the treated mice (Figure 4A). In the liver, a trend of induction of Shc activation and a significant increase in expression was detected (Figure 4B, $^{*}p < 0.05$), suggesting that the Shc activation may play a role in the progression of NASH.

3.4 | Idebenone therapeutic application improves insulin sensitivity, hepatic inflammation, and fibrosis in FFD fed mice

Shc directly binds to insulin receptors and inhibits IRS phosphorylation and its downstream signaling transduction.[4–6] We next evaluated insulin sensitivity in these mice. Glucose tolerance and insulin sensitivity improved significantly in idebenone treated mice ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{\#}p < 0.05$ compared to FFD group) (Figure 5A,B). To evaluate the downstream signaling of the insulin receptors, immortalized hepatocyte cell line FL83B cells were challenged with insulin (1 nM) with idebenone in ascending doses (Figure S2).

Akt phosphorylation in response to insulin was enhanced by idebenone in a dose-dependent fashion.

Real-time qPCR on liver tissues revealed that pro-inflammatory genes were reduced by idebenone (Figure 5C): monocyte chemoattractant protein-1 (MCP1) and C-X-C motif chemokine ligand 2 (Cxcl2) significantly decreased ($*p < 0.05$, $**p < 0.01$); tumor necrosis factor-α (TNF-α) and IL-1β showed a trend of reduction. The profibrogenic markers αSMA, col1a1, TGFβ, and Timp1 were also decreased (Figure 5D) ($*p < 0.05$). Although steatosis did not change significantly (data not shown), H&E showed fewer inflammatory patches in the treated mice (Figure 5F). Fibrosis was improved significantly as shown in hydroxyproline assay and picrosirius red staining (Figure 5E,F). These data indicated that idebenone therapeutic application attenuates inflammation and fibrosis in FFD-induced NASH.

4 | DISCUSSION

Shc is a reasonable drug target for NASH for multiple reasons. First, Shc expression rises in NASH in humans, mice, and rats.[7,9–12,15] We have shown in this study that Shc activation and expression increased in PBMCs of both MCD and FFD models; furthermore, idebenone blocked Shc activation in PBMCs (Figures 2A and 4A) confirming the target engagement. We also find a positive correlation of phospho-Shc and the serum ALT level, marker of liver injury (data not shown).

We have shown recently that idebenone directly interacts with Shc and inhibits Shc activity at the ~100 nM serum concentration that is achievable in normal human dosing.[16,23,24] This suggests that idebenone's clinical benefit is based on its inhibition of Shc rather than an antioxidant function, that is only observed at concentrations >10 μM, which have never been achieved in human dosing.

Here we evaluated the efficacy of idebenone in two models of NASH. For pharma-based drug development, the most important aspect of a NASH drug is its ability to combat terminal fibrosis late in NASH, and the MCD diet is known as a "fibrotic NASH" model.[25] Idebenone protected the mice from MCD-induced liver injury and fibrosis, demonstrated by significant reductions in serum ALT and fibrotic markers, and improved histology (Figure 3). However, this MCD model doesnt' reflect the complete phenotype of human NASH. So we tested idebenone in the FFD NASH model, which has a 'metabolic' phenotype with insulin resistance, steatohepatitis, and more mild fibrosis.[25] In this model, we used the more stringent therapeutic paradigm, in which idebenone was given late, that is, after liver injury had developed, and the major NASH parameters were improved by idebenone. Just as idebenone was previously shown to improve GTT and ITT in the corticosterone model of Type 2 Diabetes,[16] we show that idebenone therapeutic dosing significantly improves the insulin sensitivity in the FFD model (Figure 5). We also proved that idebenone enhanced insulin sensitivity in immortalized hepatocytes FL83B (Figure S2). Furthermore, liver inflammation and fibrosis were ameliorated in FFD mice (Figure 5). In general, idebenone's therapeutic effect was greater in the MCD model of NASH, in which the drug was given preventively, than in the FFD model of NASH, in which idebenone was given

later. This is to be expected and shows a significant benefit whether given during early NASH or late NASH.

Overall, our data support the idea that in NASH, Shc rises and becomes more active, and leads to insulin resistance through deactivation of phospho-Akt and the metabolic insulin response, and increased fibrosis through increased ERK/JNK/c-Jun signaling.[26] We believe these two mechanisms underlie idebenone's pro-metabolic and anti-fibrotic effects.

Genetic reduction of Shc is hepatoprotective in other liver disease contexts. Mice with genetic Shc reduction resist NASH pathophysiology caused by FFD,[12] and these same mice with genetic Shc reduction are protected from alcoholic liver injury.[27] These findings support the idea that Shc inhibition is hepatoprotective in multiple liver disease contexts including NASH and Alcoholic Liver Disease, and these could be relevant to patient populations in which to test idebenone, that already has been through >20 clinical trials for different indications.

5 | CONCLUSION

We have shown that Shc inhibitor idebenone exerts a beneficial effect in two mouse models of NASH. Shc inhibition could be a potential therapeutic strategy for NASH and other liver diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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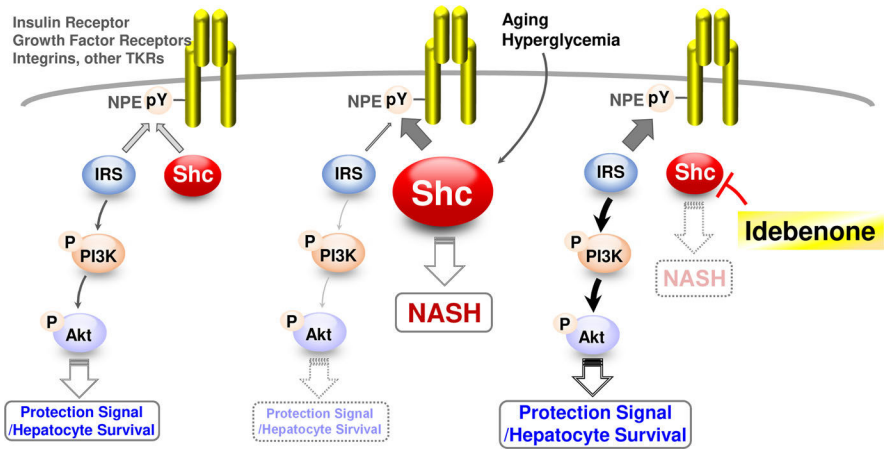

FIGURE 1.

Schematic diagram showing the working mechanism of Shc and Shc inhibitor idebenone. Shc competes with IRS for the docking site on the NPE motif ^{960}Y. Shc and IRS signaling keep balance in normal condition. Shc expression and activation are enhanced in aging and with the existence of hyperglycemia. Therefore, more inhibition will be applied onto the IRS-downstream PI3K/Akt survival signals resulting in an imbalance with impaired hepatocyte survival. This will lead to NASH progression. The imbalance can be reversed by Shc inhibitor idebenone. IRS, insulin receptor substrate; NASH, nonalcoholic steatohepatitis; PI3K, phosphoinositide 3-kinase

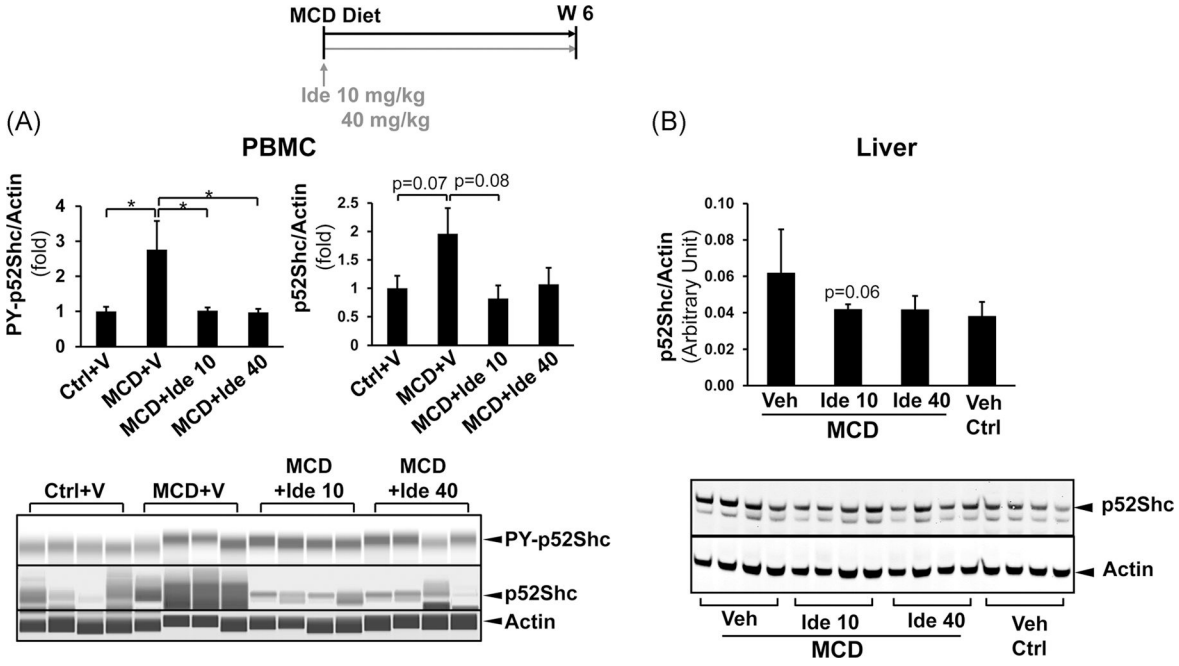

FIGURE 2.

Shc expression and activation in PBMC and liver in MCD model. Six-week-old male C57B6J mice were fed with an MCD diet for 6 weeks with idebenone 10 or 40 mg/kg oral daily starting at Day 1 of the diet representing a preventive treatment. Shc phosphorylation and activation in PBMC (A) and liver (B) were analyzed with either Jess capillary Western blot or conventional Western blot analysis. The image signal was quantified with the Compass software. Phosphorylation and expression of p52Shc increased in PBMC in the MCD group and dramatically reduced by idebenone on both 10 and 40 mg/kg. In the liver, Shc expression had a trend of reduction by idebenone, but less extent compared to PBMC. Representative images are shown; mean ± SD, $N = 4$. Ctrl, control; Ide, idebenone; MCD, methionine-choline deficient; PBMC, peripheral blood mononuclear cell. *$p < 0.05$

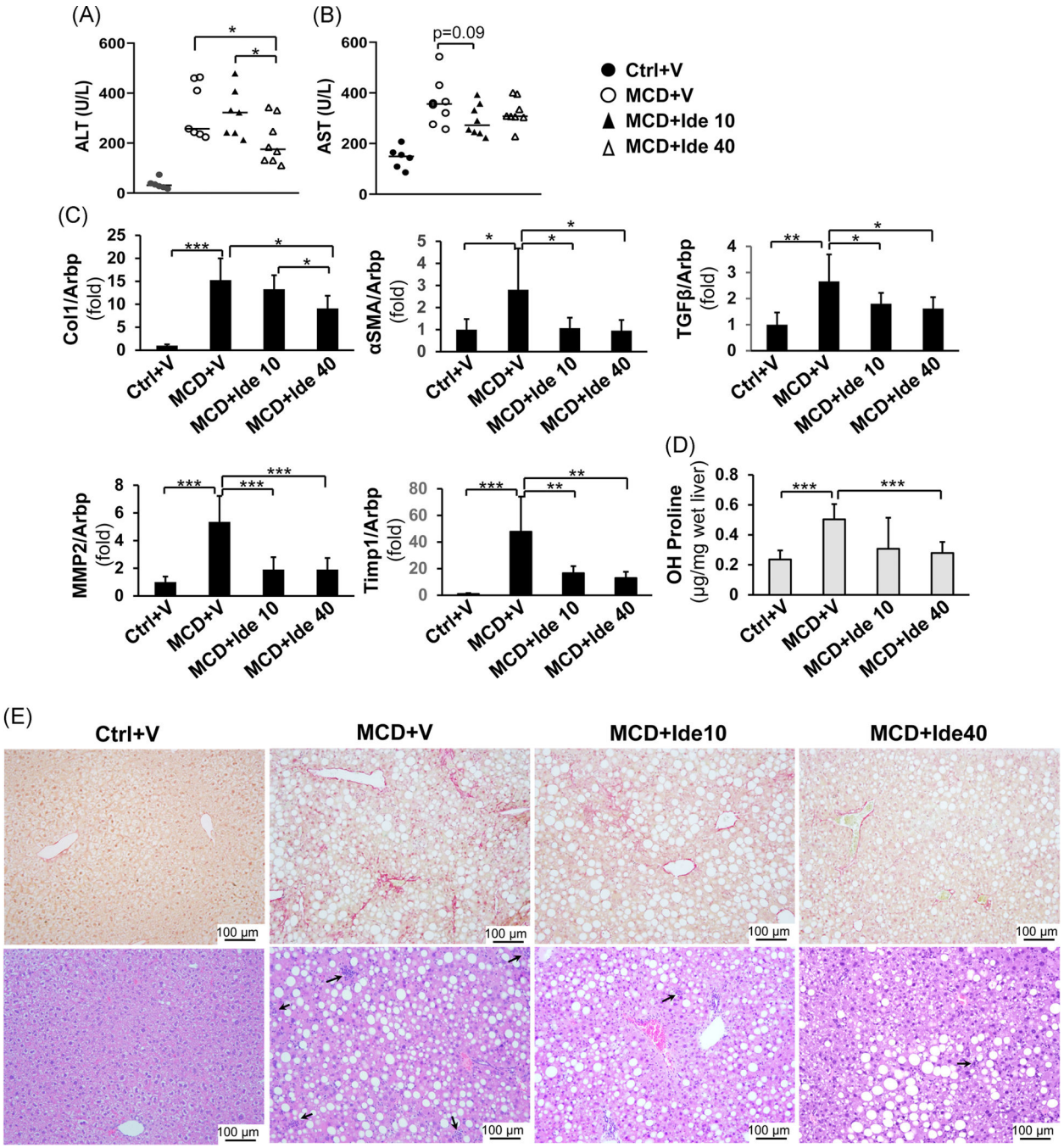

FIGURE 3.

Idebenone protects mice from MCD diet-induced liver injury. The mice were on an MCD diet with preventive idebenone dosing for 6 weeks. Serological study showed that idebenone 40 mg/kg decreased the ALT level significantly (A). AST had a trend of decrease at both 10 and 40 mg/kg (B). Real-time qPCR on liver tissues (C) showed that the idebenone reduced the transcripts of fibrogenic genes (Col1a1, αSMA, TGFβ, MMP2, and Timp1) significantly. The reduction of Col1a1 showed a dose-dependent trend. Hydroxyproline assay (D) showed a lower amount of collagen content detected in both dosage groups.

Picrosirius red staining and H&E (E) showed improved fibrosis and histology in the treated mice. Arrows indicate the patches of inflammatory cells; mean ± *SD*, $N = 8–10$. ALT, aminotransferase; AST, aspartate aminotransferase; Col1a1, collagen type I alpha 1 chain; H&E, hematoxylin and eosin; Ide, idebenone; MCD, methionine-choline deficient; MMP2, matrix metalloproteinase 2; qPCR, quantitative polymerase chain reaction; TGFβ, transforming growth factor-β; αSMA, α-smooth muscle actin. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

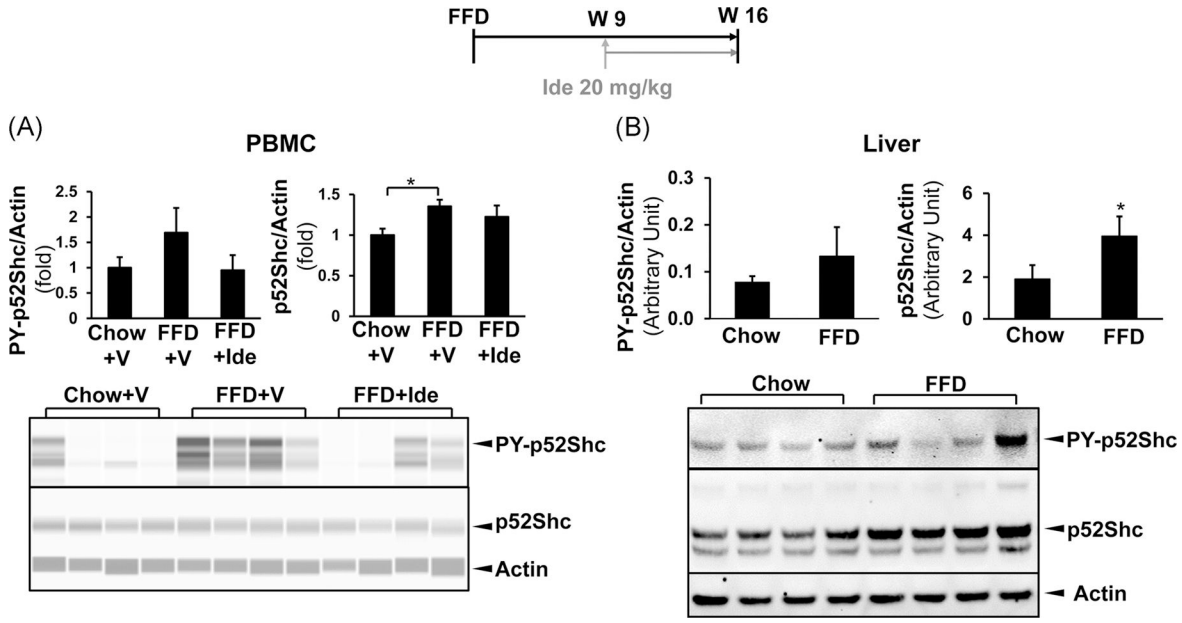

FIGURE 4.

Shc expression and activation in PBMC and liver in FFD model. Aged mice (>10 m) were on FFD for 16 weeks. Idebenone 20 mg/kg oral daily was started at the 9th week for 8 weeks long, as therapeutic dosing. PBMC was subjected to Jess capillary Western blot analysis to analyze p52Shc phosphorylation and expression (A). Liver tissues were processed for Western blot analysis (B). In PBMC, Shc phosphorylation and expression were enhanced in FFD mice. Shc phosphorylation was suppressed by idebenone. In the liver, Shc expression significantly increased, and phosphorylation showed a mild trend of increase. Representative images are shown; $N = 4$, mean ± *SD*. FFD, free-form deformation; Ide, idebenone; PBMC, peripheral blood mononuclear cell. *$p < 0.05$

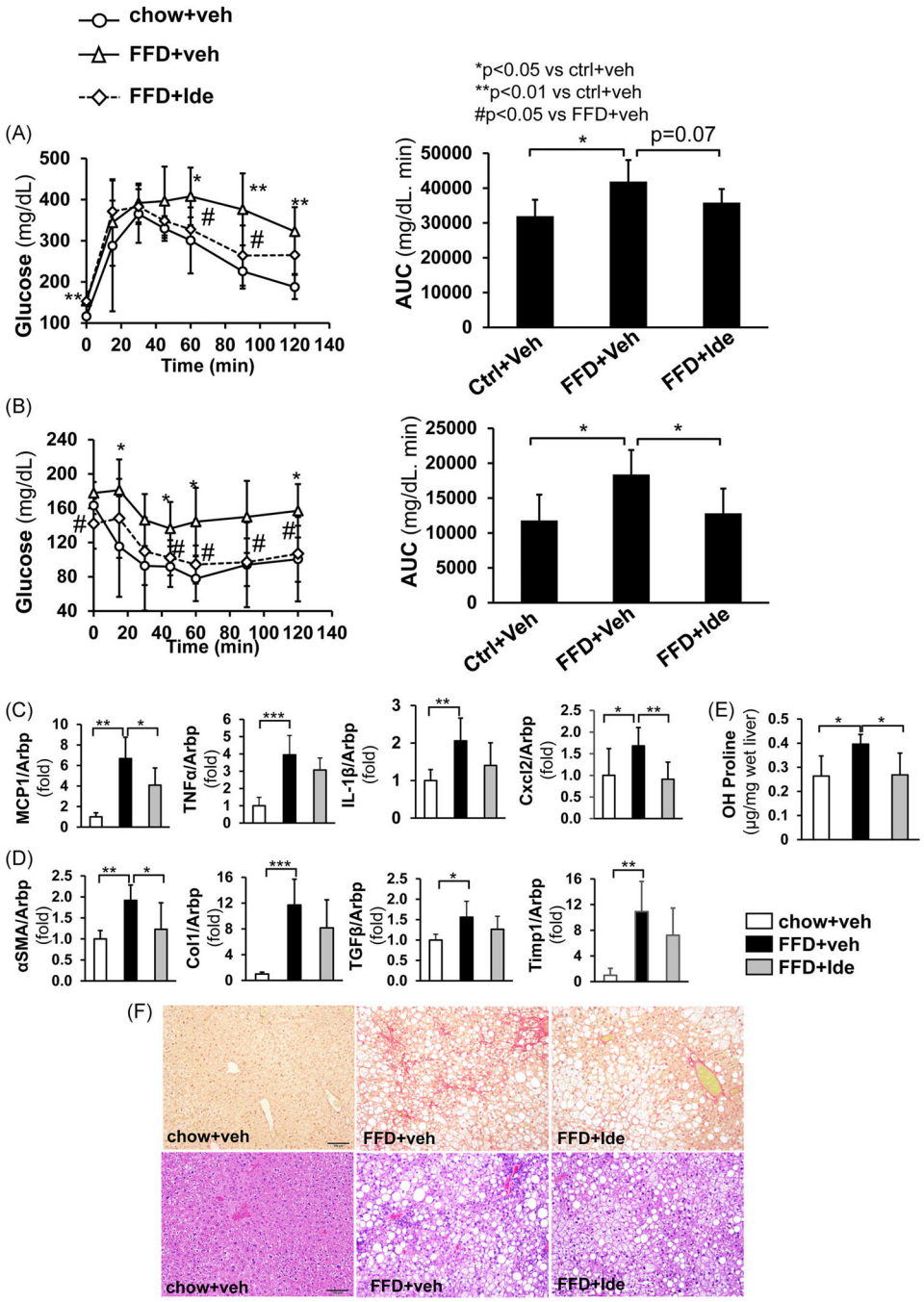

FIGURE 5.

Idebenone improves insulin sensitivity and liver injury in mice on FFD. Mice on FFD with idebenone 20 mg/kg therapeutic dosing 8 weeks were subjected for GTT (A) and ITT (B). AUC was calculated. GTT and ITT were significantly improved by idebenone. Real-time PCR on liver tissues showed reduced inflammatory markers (C: MCP1, TNF-α, IL-1β, and Cxcl2) and fibrotic markers (D: αSMA, collagen 1, TGFβ, and Timp1) in the idebenone group. Hydroxyproline quantification (E) showed significantly less collagen in these mice. Picrosirius red and H&E staining (F) showed improved fibrosis and inflammation as

well; mean ± *SD*, $N = 6–8$. AUC, area under the curve; Ctrl, control; Cxcl2, C-X-C motif chemokine ligand 2; FFD, free-form deformation; GTT, glucose tolerance test; H&E, hematoxylin and eosin; Ide, idebenone; IL-1β, interleukin-1β; ITT, insulin tolerance test; MCP1, monocyte chemoattractant protein-1; PCR, polymerase chain reaction; TGFβ, transforming growth factor-β; TNF-α, tumor necrosis factor-α; Veh, vehicle; αSMA, α-smooth muscle actin. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{\#}p < 0.05$ to FFD group

TABLE 1

Primer list

Mouse Col1a1	Forward: 5'-AGAGGCGAAGGCAACAGTCG-3'
	Reverse: 5'-GCAGGGCCAATGTCTAGTCC-3'
Mouse αSMA	Forward: 5'-TGTGCTGGACTCTGGAGATG-3'
	Reverse: 5'-CAAGTCCAGACGCATGATGG-3'
Mouse TGFβ	Forward: 5'-TTGCTTCAGCTCCACAGAGA-3'
	Reverse: 5'-CAGAAGTTGGCATGGTAGCC-3'
Mouse MMP2	Forward: 5'-ACTCCGGAGATCTGCAAACA-3'
	Reverse: 5'-ACTGTCCGCCAAATAAACCG-3'
Mouse Timp1	Forward: 5'-GTGCACAGTGTTTCCCTGTT-3'
	Reverse: 5'-GACCTGATCCGTCCACAAAC-3'
Mouse MCP1	Forward: 5'-AACTGCATCTGCCCTAAGGT-3'
	Reverse: 5'-CTGTCACACTGGTCACTCCT-3'
Mouse TNF-α	Forward: 5'-CTCATGCACCACCATCAAGG-3'
	Reverse: 5'-ACCTGACCACTCTCCCTTTG-3'
Mouse IL-1β	Forward: 5'-AGCTTCAAATCTCGCAGCAG-3'
	Reverse: 5'-TCTCCACAGCCACAATGAGT-3'
Mouse Cxcl2	Forward: 5'-TCCCTCAACGGAAGAACCAA-3'
	Reverse: 5'-AGGCACATCAGGTACGATCC-3'
Mouse Arbp	Forward: 5'-CAAAGCTGAAGCAAAGGAAGAG-3'
	Reverse: 5'-AATTAAGCAGGCTGACTTGGTTG-3'