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TRPV1 Protect against Hyperglycemia and Hyperlipidemia Induced Liver Injury via OPA1 in Diabetes

Ting Wang,[1] Yingmei Chen,[1] Yong Li,[2] Zhen Wang,[1] Chenming Qiu,[1] Dachun Yang[1] and Ken Chen[3,4]

[1]Department of Cardiology, The General Hospital of Western Theater Command, Chengdu, Sichuan, China
[2]Department of Cardiology, The People's Hospital of Chaotian District in Guangyuan, Guangyuan, Sichuan, China
[3]Department of Cardiology, Chongqing Renji Hospital, University of Chinese Academy of Sciences, Chongqing, China
[4]Department of Cardiology, The Fifth People's Hospital of Chongqing, Chongqing, China

Type 2 diabetes mellitus (T2DM)-associated mitochondrial impairment may a key factor leading to liver injury. Transient receptor potential receptor vanilloid 1 (TRPV1) regulates the energy expenditure and cholesterol metabolism in hepatocytes and protects against oxidative toxicity. Optic atrophy 1 (OPA1) is involved in the protection of TRPV1 on cardiac microvascular and lung injury. The aim of this study is to identify the role of TRPV1 in redox signals and liver protection via OPA1. TRPV1 knockout (TRPV1$^{-/-}$) mice were used. And T2DM associated liver injury was induced by high glucose and high fatty acid (HG/HF) treatment. Mechanisms were studied by TUNEL staining, transmission electron microscope (TEM) analysis, reverse transcription polymerase chain reaction (RT-PCR) and Western blotting *in vivo* and *in vitro*. We determined that HG/HF treatment increased TRPV1 expression in liver tissues and AML12 cells. The knockout of TRPV1 increased the apoptotic hepatocytes rate. The inhibition of TRPV1 by 5'-iRTX in HG/HF group elevated the reactive oxygen species (ROS) levels, whereas TRPV1 agonist capsaicin reduced ROS. Our studies also showed that the OPA1 expression was lower in livers from HG/HF treated mice than the control, and genetic ablation of TRPV1 decreased OPA1 expression to a greater extent than the HG/HF mice. The protective effects of TRPV1 on mitochondrial were blocked by OPA1 siRNA. In conclusion, our study showed that the identified regulation of TRPV1 to OPA1 has important implication to the pathogenesis of T2DM-associated liver injury. Targeting the action of TRPV1 and OPA1 presents a potential therapeutic intervention.

Keywords: mitochondria; OPA1; TRPV1; type 2 diabetes mellitus
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Introduction

Type 2 diabetes mellitus (T2DM) is one of the leading causes of death in the world, which directly causes at least 1.5 million deaths annually (Rines et al. 2016). The diabetic target organ injuries play a key role in the disease associated mortality, and diabetes also increases comorbidities of several other chronic health problems, including cardiovascular disease, stroke, and kidney disease (Hossain et al. 2007). Moreover, liver constitutes a key organ in systemic metabolism, contributing substantially to the development of insulin resistance and diabetes, which is also impaired by the hyperglycemia and hyperlipidemia in diabetes (Garcia-Compean et al. 2009; Kim et al. 2020). The hyperglycemia- and hyperlipidemia-induced hepatocellular toxicity leads to the chronic liver diseases such as non-alcoholic fatty liver disease (NAFLD), hepatic fibrosis, cirrhosis, and liver failure (Garcia-Compean et al. 2009; Mansour et al. 2019).

Various mechanisms are involved in the diabetes asso-

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Correspondence: Ken Chen, M.D., Ph.D., Department of Cardiology, Chongqing Renji Hospital, University of Chinese Academy of Sciences and The Fifth People's Hospital of Chongqing, No. 24 Renji Road, Nan'an District, Chongqing 400062, P.R. China.
e-mail: ck_tmmu@sina.com
Dachun Yang, M.D., Ph.D., Department of Cardiology, The General Hospital of Western Theater Command, No. 270 Rongdu Ave., Jinniu District, Chengdu, Sichuan 610083, P.R. China
e-mail: yangdc71@126.com

ciated liver injury. Hyperglycemia and hyperlipidemia lead to mitochondria dysfunction which causes cellular energy depletion and reactive oxygen species (ROS) generation and finally results in hepatocellular apoptosis and death (Gouaref et al. 2017; Tilg et al. 2017). Previous studies demonstrated that the transient receptor potential receptor vanilloid 1 (TRPV1) is a mechanosensitive ion channel that has been shown to regulate the energy expenditure and cholesterol metabolism in hepatocytes (Wang et al. 2013; Harb et al. 2019), while activation of TRPV1 has been identified to protect against stresses induced oxidative toxicity in various cells such as neuronal cells (Ataizi and Ertilav 2020), cardiomyocytes (Oncel and Ovey 2019), human umbilical artery smooth muscle cells (Schwartz et al. 2018) and bone marrow-derived macrophages (Yan et al. 2019). Therefore, we infer that TRPV1 could modulate redox signals to prevent high glucose- and high fatty-acid-intake-induced liver injury. And the mechanisms underlying TRPV1 lowering ROS levels are still unclear. Previous studies showed optic atrophy 1 (OPA1) is involved in the protection of TRPV1 on cardiac microvascular injury (Li et al. 2018) and lung inflammation (Xu et al. 2019; Wang et al. 2019). Here, we demonstrate that TRPV1 protect against hyperglycemia- and hyperlipidemia-induced liver injury via OPA1.

Materials and Methods

Animals

Adult male C57BL/6J mice (6-8 weeks old) were obtained from Vital River (Beijing, China), while TRPV1$^{-/-}$ mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Mice were housed under a 12 light-dark cycle at 25˚C and given free access to standard rodent food and tap water. Mice were randomized into control and high glucose- and high fatty acid (HG/HF)-induced type 2 diabetes mellitus (T2DM) groups. The T2DM mice were induced by 8-week HG/HF feeding and low-dose streptozotocin injection (intraperitoneal injection, 30 mg/kg/d, with 8-h fasting, for 5 consecutive days; Sigma-Aldrich, St. Louis, MO, USA), while mice were fed normal standard chow with intraperitoneal injection of citrate buffer as control.

This study was approved by the Research Council and Animal Care and Use Committee of The General Hospital of Western Theater Command. All animal experiments were conformed to the guidelines of the American Association for the Accreditation of Laboratory Animal Care and conformed to the guidelines of the ethical use of animals and all efforts were made to minimize suffering to reduce the number of animals used.

Cell culture

The mice normal liver cell line, AML12 cells, was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml selenium and 40 ng/ml dexamethasone at 37˚C with 5% CO_2. AML12 cells were treated with high-glucose medium (HG, 25 mmol/L) and high fatty acid (HF, 0.66 mmol/L oleic acid and 0.33 mmol/L palmitic acid; Sigma-Aldrich, Merck KGaA) for 24 h, and normal glucose medium (5.5 mmol/L) was used as control. For pharmacological interventions, the cells were then treated with TRPV1 antagonist 5-iodo-resiniferatoxin (5'-IRTX, 1 μmol/L; Abcam, Cambridge, United Kingdom) or TRPV1 agonist capsaicin (Cap, 1 μmol/L; Abcam) with HG/HF for 24 h.

OPA1 specific siRNA and Scramble siRNA (OPA1 siRNA: 5'-AAGTTATCAGTCTGAGCCAGGTTdTdT-3'; scrambled siRNA sequence: 5'-TTCGATGCCAGTCGTGCdTdT-3') were transfected in cells with 6 μL of oligofectamine in Optimem medium (Invitrogen Life Technologies) for 24 h and cultured in DMEM and Ham's F12 medium supplemented with 10% FBS for another 24 h at 37˚C with 5% CO_2. The effect of siRNA was checked by immunoblotting.

TUNEL staining

The apoptotic levels of liver were tested by TUNEL assay (Beyotime Institute of Biotechnology, Shanghai, China). WT and TRPV1$^{-/-}$ liver tissues were fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. The sections (4 μm) were stained by TUNEL kits. The result was shown as the percentage of TUNEL-positive cell nuclei in total nuclei.

Reactive oxygen species (ROS) level detection

ROS productions of AML12 cells were evaluated by the fluorescent dye dihydroethidium (DHE; Molecular Probes, Eugene, OR, USA) staining. Cells grown on coverslips were stained with 5μM DHE at 37℃ for 30 min. The images were taken with an Olympus BX51 Fluorescence Microscope (Olympus America Inc, Center Valley, PA, US). And ROS levels were also checked by lucigenin-enhanced luminescence assay (Beyotime Institute of Biotechnology).

Superoxide dismutase (SOD) activity detection

AML12 cells (1×10^6 per well) were plated in 6-well plates and lysed in lysis buffer (Beyotime Institute of Biotechnology). SOD activity measurement was performed by the assay kit (Beyotime Institute of Biotechnology) and detected by the absorbance at 450 nm using a microplate reader (Model 680; Bio-Rad, Hercules, CA, USA). The results were expressed in U/mg protein.

Mitochondrial membrane potential (MMP) assay

JC-1 kits (Invitrogen, Carlsbad, CA, USA) were used to check the MMP levels. After incubating with an equal volume of JC-1 staining solution (10 μg/ml) for 20 min at 37℃ in the dark, the fluorescence intensity value of cells that cultured in 24-well plates was detected by the spectro-

fluorometer (Spectra Max, Atlanta, GA, USA) with an excitation wavelength of 490 nm and emission wavelengths of 530 and 590 nm, which were expressed as ratios of emission at 590 to emission at 530 nm.

Quantification of Intracellular ATP

After lysis and centrifugation, the ATP contents in AML12 cells (1×10^4) were tested by a luciferase-based ATP assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions.

Transmission electron microscope (TEM) analysis

TEM analysis was performed for mitochondrial morphology observation. The cells (1×10^6) were collected, centrifuged (1,000 rpm, 10 min) and fixed in 3% glutaraldehyde at 4°C for 2 hours. After postfixing for 1 hour in 1% osmium tetroxide, AML12 cells were dehydrated in graded alcohols and acetones. After staining with uranyl acetate and lead citrate, the cells were photographed with electron microscope (JEM-1400, Jeol, Tokyo, Japan).

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

After extracting by Trizol (Tiangen, Beijing, China), a total 2 μg of RNA from AML12 cells and liver tissues was used to synthesize cDNA and served as a template for amplification of TRPV1 and OPA1. The primers are described as the previous report (Li et al. 2018). The RT-PCR kits (RR086A; TaKaRa, Kusatsu, Japan) were used for amplification. The relative amount of OPA1 and TRPV1 mRNA was quantitated by $2^{-\Delta\Delta CT}$ and normalized by the expression of GAPDH (forward 5'-AGGTCGGTGTGAACGGATTTG-3' and reverse 5'-TGTAGACCATGTAGTTGAGGTCA-3'). Each sample was analyzed in triplicate.

Western blotting

After washing twice with PBS, the liver tissues or AML12 cells were lysed by lysis buffer (Beyotime Institute of Biotechnology). The sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed to separate homogenates which then were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline with Tween 20 (TBST) buffer for 1 hour, and incubated with the primary antibodies, such as rabbit anti-TRPV1 (1:500; Abcam) and OPA1 (1:500; Abcam), overnight at 4°C. Then, the goat anti-rabbit secondary antibody (1:5,000, Jackson ImmunoResearch Laboratory, West Grove, PA, USA) conjugated to horseradish peroxidase was incubated with blots to check the primary antibodies, and the bands were visualized using a super signal chemiluminescence detection kit (Thermo Scientific, Waltham, MA, USA) and quantified by Image-Pro Plus 6.0 (Media Cybernetics, Bethesda, MD, USA). The amount of protein transferred onto the membranes was verified by immunoblotting for GAPDH (1:3,000; Cell Signaling, Danvers, MA, USA).

Statistical analysis

SPSS 22.0 statistics software (IBM SPSS Inc., Chicago, IL, USA) was uses for statistical analyses. The data are expressed as mean ± standard error (SE). Comparison within groups was made by ANOVA for repeated measures (or independent t-test when only 2 groups were compared). The least-significant difference (LSD) was used for post hoc test. A value of $P < 0.05$ was considered significant.

Results

HG/HF increased the TRPV1 expression in liver tissues and AML12 cells

Our study firstly checked the TRPV1 expression in liver tissues from the HG/HF intake-induced T2DM mice and HG/HF-treated AML2 cells. The mRNA and protein expression of TRPV1 in liver tissues from the HG/HF intake-induced T2DM mice were significantly increased as compared with control mice (Fig. 1A, B). And the similar observations were made with the AML12 cells subjected to HG/HF administration (Fig. 1C, D).

Inhibition of TRPV1 aggravates liver cell injury induced by HG/HF

To further elucidate the physiological role of TRPV1 in liver protection, we used a HG/HF-induced T2DM mouse model with genetic ablation of *trpv1*. In normal physiologic conditions, there are no significant differences in apoptosis levels between wild-type and $TRPV1^{-/-}$ mice, whereas the apoptotic hepatocyte rate was significantly increased in T2DM $TRPV1^{-/-}$ mice (Fig. 2A).

Moreover, the known TRPV1 antagonist and agonist, 5'-iRTX (Madasu et al. 2016) and capsaicin (Szabados et al. 2020), were used to treated AML12 cells with HG/HF administration. The data showed the DHE fluorescent intensity in AML12 cells subjected to HG/HF plus 5'-iRTX treatment was elevated and capsaicin significantly reduced ROS in HG/HF-injured cells (Fig. 2B). Similar observations were made with AML12 cells by a ROS measurement assay (Fig. 2C). And the activity of the antioxidant, SOD, was reduced by HG/HF treatment. The inhibition of TRPV1 in HG/HF group decreased SOD activity to a greater extent than the HG/HF-treated cells. Capsaicin treatment significantly increased the SOD activity (Fig. 2D).

OPA1 is involved in the protection of TRPV1 on HG/HF-induced liver injury

Since OPA1 has been recognized to be regulated by TRPV1 to protect against HG/HF injury (Li et al. 2018), we then measured the OPA1 expression in liver tissues. The TRPV1 knockout was identified by PCR and immunoblot (Fig. 3A, B). The OPA1 mRNA and protein expressions

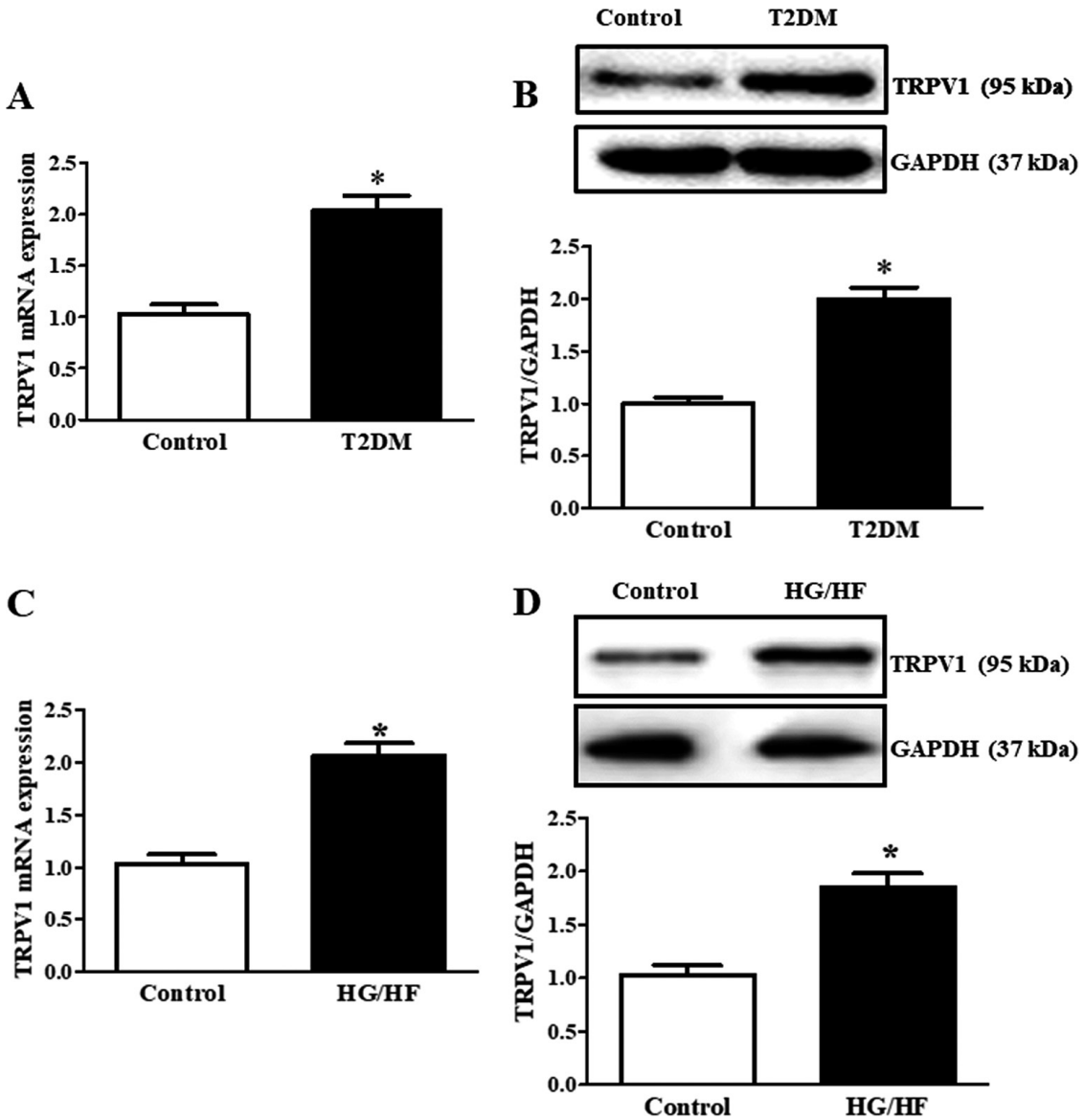

Fig. 1. The expression of TRPV1 in liver tissue from T2DM mice, and high glucose and high fatty acid (HG/HF)-treated AML12 cells.
The TRPV1 mRNA (A and C) and protein (B and D) expression in liver tissue from T2DM mice (A and B) and 24-hour HG/HF treated AML12 cells (C and D) were detected by RT-PCR and immunoblotting (*P < 0.05, vs. control, n = 6).

were lower in liver tissues from HG/HF-induced diabetes mice than the control, and genetic ablation of TRPV1 decreased OPA1 expression to a greater extent than the HG/HF-induced diabetes mice (Fig. 3C, D).

To further explore the contribution of OPA1 in TRPV1-mediated protection on HG/HF injured liver, we used siRNA to decrease the OPA1 expression in AML12 cells (Fig. 4). The electron microscopy analysis showed HG/HF led to a marked swollen and disruption of cristae of mitochondrial in AML12 cells. Capsaicin alleviated the HG/HF-induced mitochondrial injury, and the protective effects of TRPV1 on mitochondrial were blocked by OPA1 siRNA (Fig. 5A). The mitochondrial membrane potential (MMP) and ATP production were decreased in HG/HF-injured cells, and capsaicin increased MMP and ATP production in AML12 cells subjected to HG/HF treatment (Fig. 5B, C). We also found that HG/HF treatment induced ROS elevation, as shown by DHE staining and ROS measurement assay (Fig. 5D, E). In the presence of OPA1 siRNA, the protective effect of TRPV1 was lost (Fig. 5B-E).

Discussion

In this study, the data indicated that activation of TRPV1 prevents the diabetes associated liver injury through preservation of mitochondrial function via OPA1. We determined that HG/HF treatment increases TRPV1 expression. TRPV1 protects liver from HG/HF treatment via reduction of ROS activity and mitochondria-dependent apoptosis. Further studies show that OPA1 is involved in

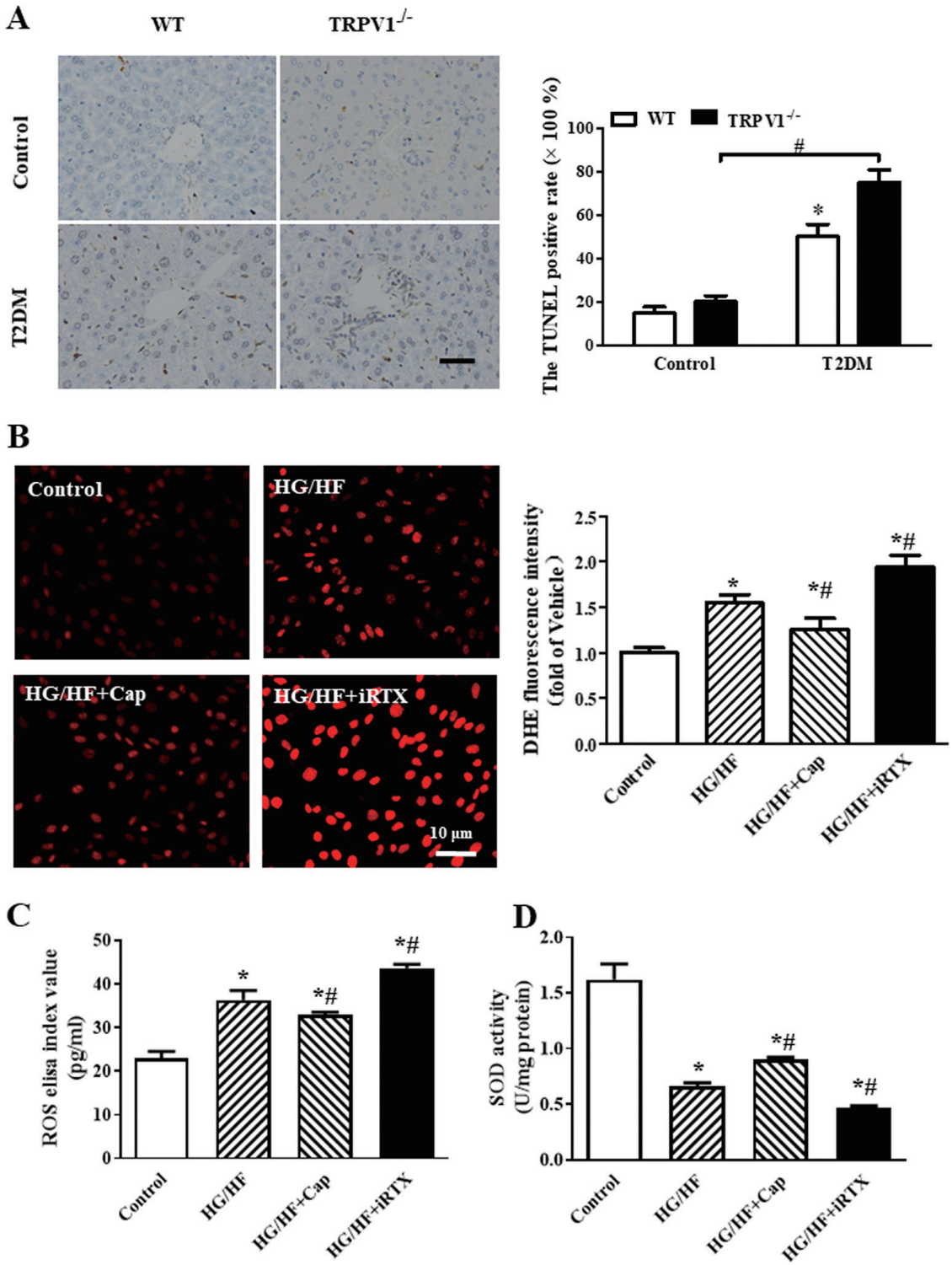

Fig. 2. Protective effects of TRPV1 against apoptosis and oxidative stress in liver tissue from T2DM mice, and high glucose and high fatty acid (HG/HF)-treated AML12 cells.

A: The cell apoptosis was tested by TUNEL staining in liver tissues. The number of positive staining cells was calculated by ImageJ software. The staining is repeated at least four times (*P < 0.05, vs. TRPV1$^{-/-}$ T2DM mice, #P < 0.05, vs. TRPV1$^{-/-}$ control mice, n = 4, scale bar = 20 μm).

B and C: The extent of ROS production in AML12 cells was determined by DHE staining (B) and lucigenin-enhanced luminescence assay (C) (*P < 0.05, vs. control, #P < 0.05, vs. HG/HF group; n = 6, scale bar = 10 μm).

D: The SOD activation was checked by a fluorescence substrate kit (*P < 0.05, vs. control, #P < 0.05, vs. HG/HF group, n = 6).

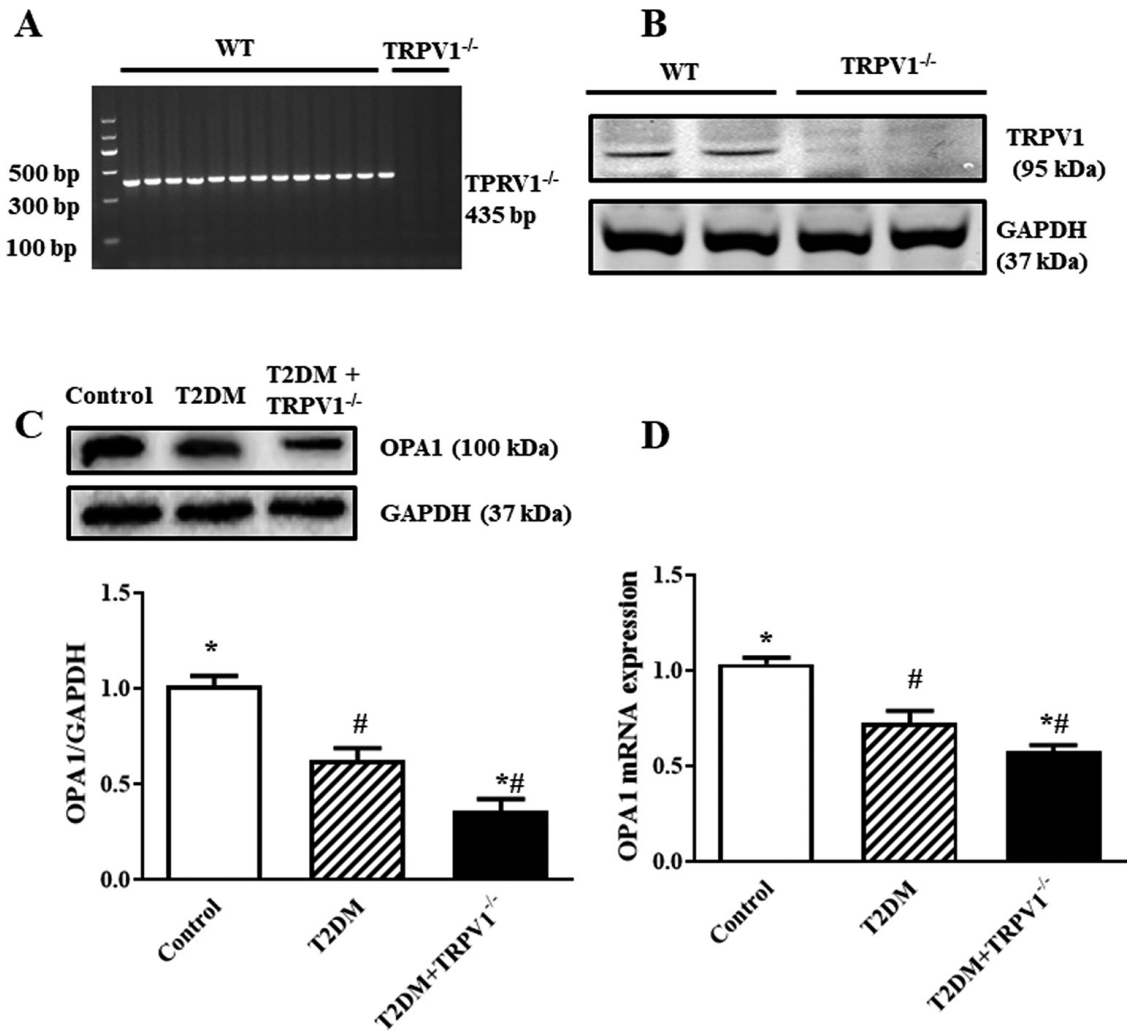

Fig. 3. The expression of OPA1 in liver tissue from T2DM mice with or without TRPV1.
A and B: The TRPV1 knockout was identified by PCR (A) and immunoblot (B).
C and D: The OPA1 protein (C) and mRNA (D) expression in liver tissue from T2DM mice were detected by RT-PCR and immunoblotting (*P < 0.05, vs. control, #P < 0.05, vs. T2DM, n = 6).

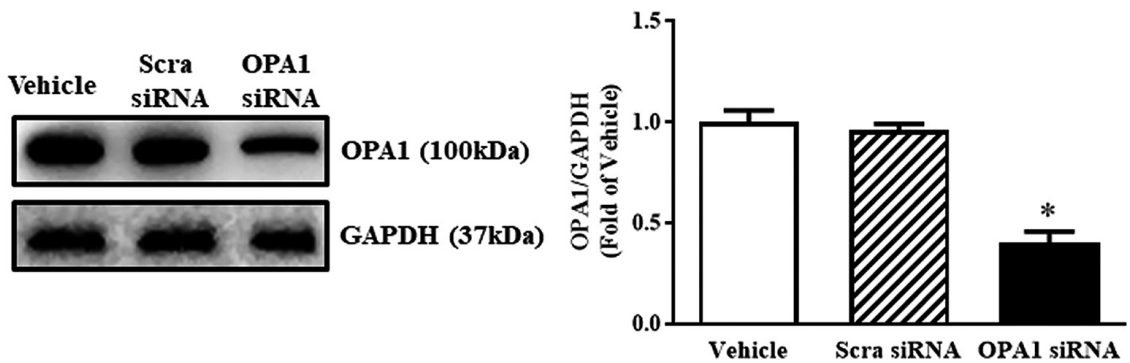

Fig. 4. The inhibitory effect of siRNA was determined by immunoblotting (*P < 0.05 vs. others, n = 4).

the regulation of TRPV1 to mitochondrial function.

There is increasing evidence that diabetes-associated hepatic injury plays an important role in liver dysfunction and metabolism disorder, and might leads to NAFLD, hepatic fibrosis, cirrhosis, liver failure and so on (Garcia-Compean et al. 2009; Mansour et al. 2019). The high glucose and high fat (HG/HF) diet could be risk factors of diabetes, which might also worsen the metabolic conditions by elevating oxidative stress on liver under the diabetic condition (Wei et al. 2020; Abo et al. 2020). Capsaicin, the

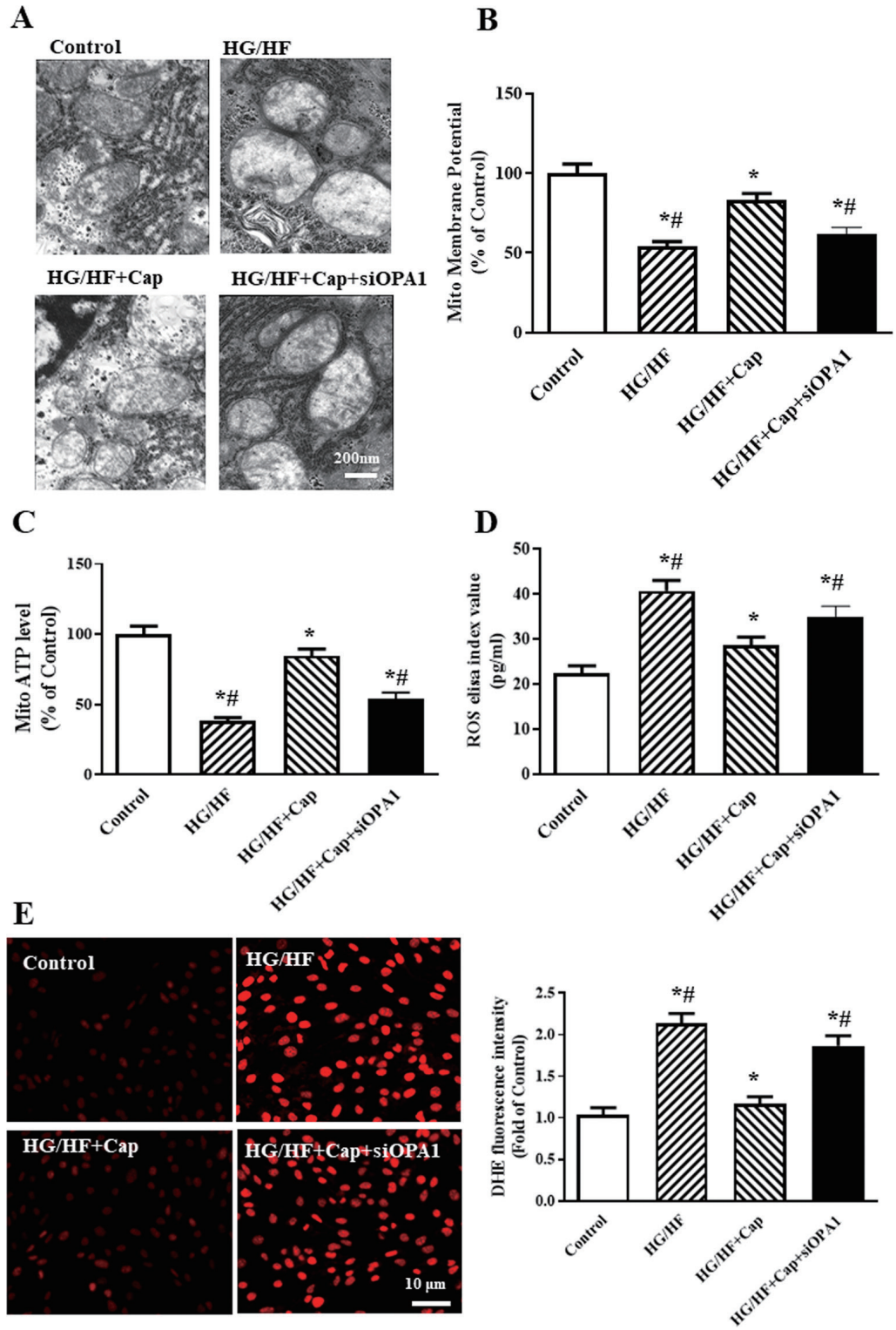

Fig. 5. OPA1 is involved in the protective effect of TRPV1.

A: The mitochondrial morphology was observed by electron microscopy. HG/HF led to a marked swollen and disruption of cristae of mitochondrial in AML12 cells, and TRPV1 agonist, capsaicin, alleviated the mitochondrial injury whereas OPA1 siRNA blocked the protective effect of TRPV1 (scale bar = 200 nm).

B: The mitochondrial membrane potential (MMP) was evaluated by JC-1 dye staining. The result was expressed as a percentage of control (*P < 0.05, vs. control, #P < 0.05, vs. HG/HF + Cap, n = 6).

C: Mitochondrial ATP production was checked by an ATP fluorometric assay kit. The result was expressed as a percentage of control (*P < 0.05, vs. control, #P < 0.05, vs. HG/HF + Cap, n = 6).

D and E: The extent of ROS production in AML12 cells was determined by DHE staining (D) and lucigenin-enhanced luminescence assay (E) (*P < 0.05, vs. control, #P < 0.05, vs. HG/HF + Cap, n = 6, scale bar = 10 μm).

major active constituent of chilli, could modulate the metabolic disorders in diabetes via the TRPV1 (Gram et al. 2019). TRPV1 is a transmembrane cation channel of transient receptor potential vanilloid family, which prefers Ca^{2+} over Na^{+} with six putative transmembrane domains and a calcium-permeable pore region (Liao et al. 2013). TRPV1 has been identified to be expressed in hepatocytes and livers (Vriens et al. 2004; Miao et al. 2008), while the TRPV1 expression is significantly increased in the diabetic liver tissues in the present study. We further inhibited the TRPV1 by genetic ablation and pharmacological interventions, and found the inhibition of TRPV1 could increase the activation of oxidative stresses and cell apoptosis. However, the underlying mechanisms that suppression of TRPV1 result in enhanced HG/HF induced liver injury is unclear.

Published papers and our previous study both indicated mitochondrial protein OPA1 is involved in the protection of TRPV1 in various stressed injured cells (Li et al. 2018; Xu et al. 2019; Wang et al. 2019). And we also revealed TRPV1 knockout and antagonist can decrease the OPA1 expression. OPA1 is essential for efficient mitochondrial inner membrane fusion and maintains mitochondrial integrity and function under physiological conditions, whereas OPA1 dysfunction might lead to cytochrome C release and cell apoptosis (Cipolat et al. 2004; Frezza et al. 2006). Here the present data provide evidence that OPA1 siRNA could block the protective effect of TRPV1 on HG/HF injury shown as mitochondrial integrity impairment, energy depletion and free radical generation. However, the underlying mechanisms remain largely unknown. Our previous study showed the activation of OPA1 is regulated by the intracellular calcium in cardiac microvascular endothelial cells in diabetes (Li et al. 2018), while TRPV1 has been found to regulate Ca^{2+} influx (Li et al. 2018; Xu et al. 2019; Mayer et al. 2020), indicating that calcium might be involved as a signal in the regulation of OPA1 on TRPV1. Our further research will focus on the role of TRPV1 in regulation of OPA1 in the hepatocytes.

In conclusion, our study showed that the identified regulation of TRPV1 to OPA1 has important implication to the pathogenesis of diabetes-induced liver injury. One can envision that therapeutic means targeting TRPV1 and OPA1 in modulating mitochondria-dependent ROS signaling and cell apoptosis may have translational value for treating diabetes-associated liver injury.

Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.

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