Cav3.2 deletion attenuates nonalcoholic fatty liver disease in mice

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is one of the most common chronic liver diseases and also the main cause of liver cirrhosis and hepatocellular carcinoma. Cav3.2 channel is an important member of T-type calcium channel and plays a vital role in energy and metabolic balance. However, the effects of Cav3.2 on NAFLD remain unclear. Here, we aimed to investigate the function of Cav3.2 channel in the development and progression of NAFLD. After 16 weeks on a high-fat diets (HFD), Cav3.2 knockout (Cav3.2 KO) improves hepatic steatosis, liver injury and metabolic syndrome in NAFLD mice model. We provided evidence that Cav3.2 KO inhibited HFD-induced hepatic oxidative damage, inflammation and hepatocyte apoptosis. In addition, Cav3.2 KO also attenuated the hepatic lipid accumulation, oxidative damage, inflammation and hepatocyte apoptosis in palmitic acid/oleic acid (PAOA)-treated primary hepatocytes. Further, Cav3.2 KO-mediated liver protection function were dependent on its interaction with CaMKII signaling. These results suggest that therapeutic approaches targeting Cav3.2 provide effective approaches for treating NAFLD.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is a complex disease that consists of a spectrum of liver injury from simple steatosis to nonalcoholic steatohepatitis (NASH) with or without hepatic fibrosis\cite{1, 2}. NAFLD is one of the most common chronic liver diseases and also the main cause of liver cirrhosis and hepatocellular carcinoma\cite{3}. Epidemiological data show that prevalence of NAFLD globally is approximately 25% and closely related to the increased morbidity and mortality of liver diseases \cite{4, 5}. At the organ level, NAFLD is described as hepatic fat accumulation, dyslipidemia and insulin resistance. On the molecular level, NAFLD is associated with a consecutive impairment of the oxidative stress and inflammatory response\cite{6-8}.

Abnormal intracellular calcium homeostasis has been identified as an important mediator of NAFLD\cite{9}. T-type calcium channels are present in hepatocytes and as an intracellular messenger to mediate the regulation of liver metabolism\cite{9, 10}. Cav3.2 channel is an important member of T-type calcium channel and involved in many cellular processes with different cell activities by regulating calcium influx, including proliferation, migration, differentiation and apoptosis\cite{11, 12}. Numerous studies suggested that Cav3.2 channel play a crucial part in the development of various diseases, including myocardial infarction\cite{13}, hypertension\cite{14}, obesity\cite{15} and diabetic neuropathy\cite{16}. However, little is known on the function of Cav3.2 channel in the progression of NAFLD.

In the present study, we used Cav3.2 KO mice to investigate the specific function of Cav3.2 in the development of NAFLD. The results indicated that Cav3.2 KO attenuated high-fat diets (HFD)-induced NAFLD by reducing the activation of calcium/calmodulin-dependent protein kinase II (CaMKII) signaling pathway. Taken together, our results supported that therapeutic approach targeting Cav3.2 provide effective approaches for treating NAFLD.
Materials and Methods

2.1. Mice

Cav3.2 KO mice with a C57BL/6 background were obtained from Cyagen Biosciences Co., Ltd (https://www.cyagen.com/cn/zh-cn/, Suzhou, China), and wild type (WT) mice in the same brood were used as controls. Animals were maintained in a specific-pathogen-free system with free access to standard chow diet. Studies were performed in 8-week-old male WT and Cav3.2 KO mice fed either a normal control diet (NCD, 12.0% kcal fat, 67.4% kcal carbohydrates and 20.6% kcal protein) or a HFD (42.0% kcal fat, 44.0% kcal carbohydrates and 14.0% kcal protein) for 16 weeks as previously described\[17\]. Animal procedures met the standards of the Guide for the Care and Use of Laboratory Animals (NIH Publication, revised 2011) and were approved by the Animal Experimentation Ethics Committee of The First Affiliated Hospital of USTC, (approval No. 202106241827000099712).

2.2. Cells and culture

Primary hepatocytes were isolated from WT and Cav3.2 KO mice and cultured as previously described\[18\]. Briefly, the liver samples were collected from WT and Cav3.2 KO mice and digested with collagenase buffer (C4-BIOC, Sigma-Aldrich, USA) and then purified using via Percoll solutions. Primary hepatocytes were cultured in DMEM (10% FBS and 1% penicillin/streptomycin) and then incubated with a mixture containing 0.5 mmol/L palmitic acid (PA, P5585, Sigma-Aldrich, USA) and 1.0 mmol/L oleic acid (OA O1383, Sigma-Aldrich, USA) or fatty acid free BSA for 24 h to imitate NAFLD in vitro. To detect whether the function of Cav3.2 is associated with the CaMKII signaling, primary hepatocytes were performed with adenoviruses carrying CaMKII (Ad-CaMKII) or an NC sequence (Ad-NC) according to the manufacturer's instructions (OBiO Technology, China).

2.3. Biochemical analysis

Blood was sampled from mice and centrifugated to collect serum specimens. Next, serum aspartate transaminase (AST) and alanine transaminase (ALT), alkaline phosphatase (ALP), triglycerides (TG), total cholesterol (TC) and lactate dehydrogenase (LDH) were analyzed using a Beckman Coulter chemistry analyzer (AU5800, USA). In addition, the levels of TG (290-63701) and TC (294-65801) in the liver tissues or primary hepatocytes were measured according to the manufacturer's instructions (Wako Pure Chemical Industries, Japan).

2.4. Glucose tolerance test (GTT) and insulin tolerance test (ITT) test

GTT and ITT were performed as previously described\[19\]. Following 12 h fasting, mice were intraperitoneally injected with 1.5 g/kg glucose (PHR1000, Sigma Aldrich, USA) GTT or 1 U/kg insulin (I5523, Sigma Aldrich, USA) after fasting for 12 h. Next, blood samples were collected at 0 min, 30 min, 60 min, 90 min, 120 min, and 150 min after injection to determine the levels of blood glucose.
2.5. Oxidative stress detection

Liver samples were embedded in optimal cutting temperature (OCT) compound (4583, SAKURA Tissue-Tek, Japan) to freezing slice according to our previous research\cite{20}. Then, the frozen tissue sections were incubated with 8-hydroxy-2'-deoxyguanosine (8-OHdG) antibody (sc-66036, Santa Cruz, USA) following standard procedures. In addition, glutathione peroxidase (GSH-Px, S0056) and superoxide dismutase (SOD, S0101M) activities, malondialdehyde (MDA, S0131M) and NADPH oxidase (NOX, S0179) contents were detected using commercial reagent kits (Beyotime Biotechnology, China).

2.6. Tissue staining

Liver samples were collected and embedded in OCT compound (4583, SAKURA Tissue-Tek, Japan) to freezing slice. Next, the sections were incubated with Oil Red-O dye liquor to access hepatic fat accumulation. In addition, hepatocyte Oil-Red O staining were determined following standard procedures \cite{21}.

2.7. Western blot

Proteins were extracted using RIPA solution and then the concentrations were detected using BCA kis. Proteins were separated on SDS-PAGE gel and then transferred to PVDF membranes (IPVH00010; Thermo Fisher Scientific, USA). The membranes were incubated first with primary antibodies against Bcl-2 (ab182858, Abcam, USA), Bax (ab32503, Abcam, USA), p-CaMKII (ab171095, Abcam, USA), CaMKII (ab134041, Abcam, USA) and GAPDH (ab9485, Abcam, USA), and then incubated with secondary antibodies (ab288151, Abcam, USA). Finally, the protein band were visualized using an Odyssey imaging system (LI-COR, USA) and GAPDH was used as an internal control for corresponding protein expression.

2.8. qRT-PCR

Total RNA was extracted using TRIzol reagents and then subjected to the reverse transcription. Then, PCR were performed with SYBR® Premix Ex Taq™ II (RR820B, Takara Bio, China) and the targeted genes expression were analyzed using the $2^{-\Delta\Delta CT}$ method. The corresponding gene expression was normalized by expression of GAPDH and the primers were listed in Supplementary Table 1.

2.9. CaMKII activity assays

CaMKII activity of liver or primary hepatocytes samples was detected using a CaMKII assay kit (Promega Corporation, USA) according to previously described\cite{22}. Briefly, the fresh liver tissues or primary hepatocytes were lysed and added biotinylated CaMKII peptide substrate. After incubation for 15 min at 37°C, scintillation counter was used to detect the activity of calmodulin and CaMKII.

2.9. Statistical analysis

The data are presented as the means ± standard deviation. Statistical significance between two groups were compared using two-tailed $t$-test, while statistical significance among multiple groups were
compared using the One-Way ANOVA followed by Tukey post-hoc test. P < 0.05 was considered statistically significant.

Results

Cav3.2 KO improves hepatic steatosis and liver injury in HFD-fed mice

As shown in Fig. 1A, HFD-feeding resulted in a significant raise of body weight in the WT mice, and Cav3.2 KO attenuated the HFD-induced weight gain. Similarly, Cav3.2 KO mitigated the increases of liver weight and the ratio of liver weight to body weight in the HFD-treated mice (Fig. 1B-C). Cav3.2 KO further reduced liver lipid deposition and histological alterations in the HFD-treated mice (Fig. 1D). In addition, impaired hepatic function after HFD administration was also improved in Cav3.2 KO mice, as shown by the reduced ALT, AST, ALP and LDH levels (Fig. 1E-H).

Cav3.2 KO alleviates metabolic syndrome in HFD-fed mice

GTT and ITT results revealed that Cav3.2 KO markedly ameliorated HFD-induced insulin resistance (Fig. 2A-B). In addition, Cav3.2 KO significantly decreased serum and liver TG and TC content after HFD administration (Fig. 2C-F).

Cav3.2 KO relieves HFD-induced oxidative stress in liver

As shown in Fig. 1A, Cav3.2 KO significantly decreased 8-OHdG expression in liver tissues after HFD administration. In addition, Cav3.2 KO significantly relieves HFD-induced oxidative damage in liver, as characterized by the promoted SOD and GSH-Px activities, and by the reduced MDA and NOX contents (Fig. 3B-E).

Cav3.2 KO attenuates hepatic inflammation and hepatocyte apoptosis

The results revealed that HFD-feeding significantly increased the mRNA expression of inflammatory mediators in the livers, including IL-1β, IL-18, iNOS and COX-2 (Fig. 4A). Interestingly, Cav3.2 KO significantly reduced the mRNA expression of IL-1β, IL-18, iNOS and COX-2 in HFD-fed mice (Fig. 4A). The western blots result also showed that Cav3.2 KO markedly increased Bcl-2 expression and reduced Bax expression after HFD administration (Fig. 4B).

Cav3.2 KO improves lipid accumulation and oxidative damage in vitro
Consistent with the results of animal experiments, Cav3.2 KO significantly reduced LDH releases and lipid accumulation in PAOA-treated hepatocytes (Fig. 5A-D). In addition, Cav3.2 KO also reversed PAOA-induced oxidative damage in primary hepatocytes, as indicated by the promoted SOD and GSH-Px activities, and by the lowered MDA and NOX contents (Fig. 5E-H).

**Cav3.2 KO improves inflammation and hepatocyte apoptosis in vitro**

As expected, PAOA-induced increases of IL-1β, IL-18, iNOS and COX-2 were markedly attenuated by Cav3.2 KO (Fig. 6A-D). In addition, Cav3.2 KO also increased Bcl-2 expression and reduced Bax expression after PAOA administration (Fig. 6B).

**Cav3.2 regulates the CaMKII signaling**

CaMKII is a widely expressed multifunctional serine/threonine kinase and was reported to be regulated by Cav3.2 channel. Thus, we investigated whether the Cav3.2 involved NAFLD progression by regulating CaMKII signal. The results demonstrated that Cav3.2 KO significantly decreased the phosphorylation of CaMKII and the activity of CaMKII in HFD-fed mice (Fig. 7A). In addition, Cav3.2 KO also decreased the phosphorylation of CaMKII and the activity of CaMKII in primary hepatocytes after PAOA insult (Fig. 7B).

**CaMKII overexpression abrogates Cav3.2 KO-mediated protective function in vitro**

To further assess the role of CaMKII in Cav3.2 KO-mediated protective function, adenoviruses transfection was used to overexpress CaMKII in vitro. The results showed that CaMKII overexpression significantly increased LDH releases and lipid accumulation and aggravated PAOA-induced oxidative damage in primary hepatocyte (Fig. 8A-E). In addition, Cav3.2 KO-reduced lipid accumulation and oxidative damage were restored by CaMKII overexpression in primary hepatocytes after PAOA insult (Fig. 8A-E). Moreover, CaMKII overexpression abolished the anti-inflammatory and anti-apoptosis effects of Cav3.2 KO (Fig. 9A-E). These data directly indicate that Cav3.2 KO-mediated protective function were dependent on its interaction with CaMKII signaling.

**Discussion**

With dramatic modifications in diets and lifestyles, NAFLD has emerged as the most common chronic liver disease and affected more than 1.7 billion people worldwide\[^{23}\]. In addition, NAFLD is represents the main cause of liver cirrhosis and hepatocellular carcinoma, and associated with the increasing cause of liver-related mortality\[^{23, 24}\]. Clinical and epidemiological data discovered that 20 million people worldwide will eventually die of NAFLD-related liver disease and causes great health economic burden\[^{25, 26}\]. Multiple pathological processes have been identified as major culprits of NFALD, including oxidative
damage, chronic inflammation, dyslipidemia and hepatocyte apoptosis\textsuperscript{27, 28}. Therefore, drugs or genes targeting the above processes may be an effective strategy of improving NAFLD.

As a main member of the T-type calcium channel, Cav3.2 channel has been reported to widely present in tissues throughout the body, including heart, brain, liver, lung, kidney and skin\textsuperscript{12, 29}. Numerous studies suggested that Cav3.2 channel play a crucial part in the development of various diseases, including myocardial infarction\textsuperscript{13}, hypertension\textsuperscript{14}, obesity\textsuperscript{15} and diabetic neuropathy\textsuperscript{16}. In addition, Cav3.2 inhibitors mibefradil and NNC 55–0396 were proven to improve hyperglycemia and hyperlipidemia in db/db mice\textsuperscript{30}. However, little is known on the effect of Cav3.2 channel in the progression of NAFLD. In the present study, we used Cav3.2 KO mice to investigate the specific function of Cav3.2 in the development of NAFLD. Our studies showed that Cav3.2 KO attenuated HFD-induced hepatic fat accumulation, dyslipidemia and insulin resistance in vivo. In addition, Cav3.2 KO attenuates PAOA-triggered liver injury and lipid accumulation in vitro. These results indicating that Cav3.2 may be a potential target for NAFLD and related metabolic diseases.

Numerous studies have suggested that oxidative stress is a crucial factor mediating hepatic cell death and liver injury, which reflects an imbalance between reactive oxygen species (ROS) and the antioxidant system\textsuperscript{31, 32}. ROS are produced within the cells as a result of cellular metabolic activities and have physiological roles at low concentration. Under pathological conditions in NAFLD, overproduction of ROS can directly damage DNA, protein and lipid, which may be followed by hepatic cell death and apoptosis\textsuperscript{33, 34}. It is also well recognized that suppressing oxidative stress and scavenging overproduction of ROS can be very effective in improving the progression of NAFLD.

In the present study, 8-OHdG staining was performed to assess the DNA oxidation and the results showed that Cav3.2 KO significantly decreased the 8-OHdG expression in liver tissues after HFD administration. SOD and GSH-Px are key enzymatic antioxidants that plays pivotal roles in ROS scavenging\textsuperscript{35}. MDA is a lipid peroxidation products and considered as an important marker of free radical\textsuperscript{36}. NOX is a major source of ROS and participate in a variety of cell physiological processes\textsuperscript{37}. Intensive research has revealed that patients with NAFLD had markedly lower SOD and GSH-Px activities, while MDA and NOX contents was significantly exacerbated\textsuperscript{36}. Consistent with the above data, our results suggest that Cav3.2 KO significantly attenuates NAFLD-related oxidative stress damage, as evidenced by the increased activities of SOD and GSH-Px and the decreased levels of MDA and NOX. These results directly reflect that the protective effect of Cav3.2 KO is related to the inhibition of oxidative stress.

It has previously been reported that inflammation is one of the important pathological features of NAFLD and sustained inflammation can mediate hepatic fat accumulation and insulin resistance\textsuperscript{38, 39}. In addition, the inflammatory response and oxidative damage almost exist simultaneously and interact to form vicious cycle, which eventually aggravates hepatic damage\textsuperscript{36, 40}. Consistent with previous studies, HFD-feeding markedly increased the mRNA expression of IL-1\textbeta, IL-18, iNOS and COX-2, while Cav3.2 KO reduced the mRNA expression of above inflammatory mediators in HFD-fed mice. Moreover, hepatocyte
apoptosis also plays a crucial role in the progression of NAFLD. Thus, we evaluated the potential role of Cav3.2 in hepatocyte apoptosis. The results revealed that Cav3.2 KO significantly alleviated hepatocyte apoptosis in HFD-fed mice and primary hepatocytes after PAOA treatment.

As a serine/threonine-specific phosphokinases, CaMKII is respond to changes in intracellular calcium homeostasis and regulate a range of cellular functions[41]. CaMKII is activated upon binding calcium/calmodulin complex and lead to a conformational change, which exposes the kinase substrate binding sites[41,42]. Moreover, studies indicate that CaMKII pathway play a key role in retarding the inflammation and lessening the NAFLD process[43,44]. In addition, a growing body of evidence supported that Cav3.2 is required for activation of the CaMKII pathway[45]. Thus, we investigated whether the function of Cav3.2 on NFALD is associated with the CaMKII signaling. Consistent with previous reports[43,44], our results also demonstrated that phosphorylation of CaMKII and the activity of CaMKII was significantly up-regulated in the livers from HFD-fed mice and primary hepatocytes after PAOA treatment. In addition, Cav3.2 KO significantly decreased the phosphorylation of CaMKII and the activity of CaMKII, and CaMKII overexpression abolished the Cav3.2 KO-mediated protective function. These results directly reflect that Cav3.2 KO-mediated protective function were dependent on its interaction with CaMKII signaling.

In summary, the present study identified the function of Cav3.2 in the development of NAFLD. We found Cav3.2 KO alleviated HFD-induced hepatic steatosis and liver injury by inhibiting oxidative damage, inflammation and hepatocyte apoptosis. Further, Cav3.2-mediated effects were related to its interaction with CaMKII signaling. These results suggest that therapeutic approaches targeting Cav3.2 provide effective approaches for treating NAFLD.

**Declarations**

**Ethics approval and consent to participate**

Animal procedures met the standards of the Guide for the Care and Use of Laboratory Animals (NIH Publication, revised 2011) and were approved by the Animal Experimentation Ethics Committee of The First Affiliated Hospital of USTC, (approval No. 202106241827000099712).

**Availability of data and material**

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

**Competing interests**

Not applicable.

**Conflicts of interest**
No conflicts of interests are declared by the authors.

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**Author contributions**

LJ, TL and CT performed the major experiments, data analysis, and manuscript writing. XL, CH, FD, ZZ, CL, WZ, JW and HC designed the experiments and performed data analysis. All authors contributed to the study conception and agreed to submit this manuscript.

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**Figures**

**Figure 1**

*Cav3.2 KO improves hepatic steatosis and liver injury in HFD-fed mice.* (A) Body weight of WT and Cav3.2 KO mice (n = 12). (B-C) The liver weight and the liver weight/body weight ratio (n = 12). (D) Oil-Red-O
staining of liver samples (n = 6). Scale bar, 50 μm. (E-H) Serum ALT, AST, ALP and LDH level (n = 10). *P < 0.05 vs. NCD group; #P < 0.05 vs. HFD group.

Figure 2

Cav3.2 KO improves metabolic syndrome in HFD-fed mice. (A-B) GTT and ITT test (n = 8). (C-D) Serum TC and TG levels (n = 8). (E-F) Liver TC and TG levels (n = 8). *P < 0.05 vs. NCD group; #P < 0.05 vs. HFD group.
Figure 3

Cav3.2 KO alleviates HFD-induced oxidative damage in liver. (A) 8-OHdG staining of liver sections (n = 5). Scale bar, 50 μm. (B-E) Measurements of SOD, GSH activities, MDA and NOX levels in the liver samples (n = 5). *P < 0.05 vs. NCD group; #P < 0.05 vs. HFD group.

Figure 4
Cav3.2 KO attenuates hepatic inflammation and hepatocyte apoptosis. (A-D) RT-qPCR analysis for IL-1β, IL-18, iNOS and COX-2 in liver tissues (n = 6). (E) Western blot analysis for Bcl-2 and Bax expression in liver samples (n = 4). *P < 0.05 vs. NCD group; #P < 0.05 vs. HFD group.

Figure 5

Cav3.2 KO improves lipid accumulation and oxidative damage in vitro. (A) LDH releases (n =6). (B-C) Cellular TC and TG levels (n =6). (D) The density of lipid content (n =6). (E-H) Measurements of SOD, GSH activities, MDA and NOX levels (n = 6). *P < 0.05 vs. BSA group; #P < 0.05 vs. PAOA group.
Figure 6

**Cav3.2 KO improves inflammation and hepatocyte apoptosis in vitro.** (A-D) RTqPCR analysis for IL-1β, IL-18, iNOS and COX-2 in PAOA-induced hepatocytes (n = 6). (E) Western blot analysis for Bcl-2 and Bax expression in PAOA-induced hepatocytes (n = 4). *P < 0.05 vs. BSA group; #P < 0.05 vs. PAOA group.
Figure 7

Cav3.2 regulates the CaMKII signaling. (A) Western blot analysis for CaMKII and p-CaMKII protein in liver samples (n = 4). (E) Western blot analysis for CaMKII and p-CaMKII protein in PAOA-induced hepatocytes (n = 4). *P < 0.05 vs. BSA group; #P < 0.05 vs. PAOA group.
**Figure 8**

Effects of CaMKII overexpression on PAOA-induced lipid accumulation and oxidative damage in vitro. (A) LDH releases (n = 6). (B-C) Cellular TC and TG levels (n = 6). (D) The density of lipid content (n = 6). (E-H) Measurements of SOD, GSH activities, MDA and NOX levels (n = 6). *P < 0.05 vs. PAOA+WT+Ad-NC group.

![Graph A](image1)

**Figure 9**

Effects of CaMKII overexpression on PAOA-induced inflammation and hepatocyte apoptosis in vitro. (A-D) RTqPCR analysis for IL-1β, IL-18, iNOS and COX-2 in PAOA-induced hepatocytes (n = 6). (E) Western blot analysis for Bcl-2 and Bax expression in PAOA-induced hepatocytes (n = 4). *P < 0.05 vs. PAOA+WT+Ad-NC group.

![Graph E](image2)

**Supplementary Files**

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