

DPP-4 inhibition resembles exercise in preventing type 2 diabetes development by inhibiting hepatic protein kinase C ϵ expression in a mouse model of hyperinsulinemia

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Abstract

Objective: Interventions for hyperinsulinemia (HINS), an early indicator of type 2 diabetes mellitus (T2DM), can significantly reduce the T2DM risk. This study aims to determine how dipeptidyl peptidase-4 (DPP-4) inhibition prevents HINS progression to T2DM through ameliorating hepatic steatosis.

Methods: KK α mice were used as a HINS model and they underwent exercise or received a DPP-4 inhibitor, MK0626. Hepatic steatosis was examined and liver diacylglycerol levels were determined. Human hepatic cells (LO2) were treated with MK0626 or transfected with DPP-4 siRNA. Protein kinase C ϵ isoform (PKC ϵ) and DPP-4 expression and insulin receptor substrate 1 (IRS-1) phosphorylation were assessed using immunohistochemistry and western blot.

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Results: KKay mice developed HINS spontaneously at 7 weeks of age. Similar to exercise, MK0626 ameliorated hepatic steatosis and reduced the liver triglyceride and diacylglycerol content. Both exercise and MK0626 suppressed diacylglycerol-induced PKC ϵ expression and restored insulin signaling, which was shown by tyrosine phosphorylation of IRS-1, in the livers of KKay mice. Additionally, silencing DPP-4 or MK0626 treatment decreased PKC ϵ expression in LO2 cells.

Conclusions: Our data demonstrate that DPP-4 inhibition resembles exercise and effectively delays T2DM onset by suppressing hepatic PKC ϵ expression in the HINS mouse model.

Keywords

DPP-4 inhibitor, hyperinsulinemia, type 2 diabetes, hepatic steatosis, PKC ϵ , hepatic steatosis

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Introduction

Overeating and a sedentary lifestyle are the primary drivers causing the global epidemic of diabetes, and they result in an increased mortality rate and disability burden worldwide.¹ There were 463 million adult diabetes patients globally in 2019, and the number of patients is increasing rapidly.² Type 2 diabetes mellitus (T2DM), which is characterized by insulin resistance, accounts for 90% of diabetes cases. T2DM usually develops from impaired glucose tolerance (IGT) or impaired fasting glucose (IFG), which is called prediabetes.¹ Prediabetes influences a huge number of people in both developing and developed countries. For example, it is estimated that 493.4 million Chinese adults and 84.1 million American adults have prediabetes. Interventions can dramatically lower the risk of T2DM in prediabetic patients.¹

Hyperinsulinemia (HINS), which is defined as elevated circulating insulin without hypoglycemia, precedes prediabetes and may be exacerbated by insulin resistance.³⁻⁵ This is a compensatory response to the deficiency of insulin signaling and abnormal levels of blood glucose and a primary defect of an unhealthy diet and lifestyle.^{4,5} It is well established that enhanced insulin

signaling promotes lipogenesis and increases the delivery of fatty acids to the liver,^{6,7} which could be the causes of HINS-induced non-alcoholic fatty liver disease (NAFLD).^{4,5} Hepatic lipid accumulation can lead to hepatic insulin resistance and T2DM in the future.⁶⁻⁸

Exercise and pharmacological interventions can effectively reduce the risk of T2DM in prediabetic patients.¹ However, interventions at the HINS stage may result in a better outcome because it occurs earlier than prediabetes. Exercise can reduce lipid accumulation in the liver and reverse NAFLD.⁹ The beneficial effects of exercise on NAFLD may be mediated by multiple molecular mechanisms, such as by reducing fat acid synthesis via activation of 5'-AMP-activated protein kinase (AMPK) and by increasing fatty acid β -oxidation in hepatocyte mitochondria via activating peroxisome proliferator-activated receptors alpha (PPAR α).⁹ For people who have a physical impairment or who cannot adhere to lifestyle changes, pharmacological interventions are available options. Metformin and pioglitazone were shown to reduce the incidence of diabetes in prediabetic people.^{10,11} However, metformin is associated with gastrointestinal symptoms, and pioglitazone is

associated with significant weight gain and edema. Therefore, additional pharmacological intervention options need to be explored.

Dipeptidyl peptidase-4 (DPP-4) is a transmembrane peptidase that is widely distributed in the body, and it has a high expression in the liver.¹² DPP-4 inhibition has been used widely to lower blood glucose in T2DM patients because the inhibitors are well tolerated and have no evident adverse effects.^{13,14} DPP-4 inhibitors exert their anti-hyperglycemic function primarily by preventing the degradation of the incretins, GLP-1 and GIP, which are two peptides that promote insulin secretion.¹³ Glucose homeostasis maintenance by DPP-4 inhibitors is at least partially mediated by the liver. Hepatic DPP-4 levels are consistently associated with hepatic steatosis and insulin sensitivity.^{12,13,15} Moreover, knockout DPP-4 in mice or DPP-4 inhibition effectively reduced hepatic steatosis in both humans and mice.^{12,13} In diabetic mice, DPP-4 inhibition prevented diet-induced hepatic steatosis.¹⁶ Moreover, the enhancement of insulin resistance and induction of NAFLD that resulted from liver-specific DPP-4 expression demonstrated the role of DPP-4 in the liver.¹⁷ However, DPP-4 inhibitors have not yet been thoroughly examined to see if they can be used as a protective drug in prediabetic patients. No conclusions have been drawn based on the limited studies.^{18,19} We have previously shown that, similar to exercise, DPP-4 inhibition can significantly reduce the risk of T2DM in mice with HINS by improving pancreatic islet function.²⁰ To provide further evidence for using DPP-4 inhibitors as a protective drug, investigation into the hepatic role of DPP-4 inhibitors in mice with HINS is required. In our published study, we had shown that the FBG levels of KN and KHF mice were considerably higher compared with the CN mice at week 15 (after the intervention). The FBG levels of KE and KD mice were similar to

the CN group at week 15 and lower than 16.7 mmol/L, although the FINS levels of KE and KD groups were still significantly higher compared with the CN group at week 15. These results indicated exercise and DPP-4 inhibition prevented these mice from progressing into T2DM, and they were still in the HINS phase after the interventions ended.²⁰

HINS could induce hepatic steatosis, which causes future hepatic insulin resistance. Recent studies have shown that lipid-induced hepatic insulin resistance is not attributed to the hepatic triglyceride (TG), which is a hallmark of NAFLD, but that it was consistently associated with the elevation of diacylglycerol (DAG), a precursor of TG.^{8,21} DAG activates the ϵ isoform of protein kinase C (PKC ϵ), which in turn phosphorylates Thr1160 of the insulin receptor; this results in an insulin signaling blockade.^{7,22} However, the links between DPP-4 inhibition and DAG-induced PKC ϵ activation are currently unknown. In this study, we hypothesize that DPP-4 inhibition can resemble exercise in the improvement of hepatic insulin resistance by ameliorating hepatic steatosis and suppressing DAG-induced PKC ϵ expression in the liver. We examined this hypothesis in mice with HINS.

Materials and Methods

Animals

Four-week-old specific-pathogen-free (SPF) male C57B6/6J (C57) and KK^{ay} mice were purchased from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences (Beijing, China). All the animals were maintained in a controlled environment at a room temperature of 22 to 24°C, humidity of 40% to 60%, and a light/dark cycle of 12/12 hours. The mice had access to food and water *ad libitum*. They were acclimatized to the animal

facility at the Chinese PLA General Hospital for 1 week before the experiment. All the animal procedures were performed in accordance with “Ethical principles and guidelines for experimental animals” of the Animal Care. The protocol was approved by the Ethics Committee of Chinese PLA General Hospital, Beijing (No. DW20100139, Date: 2010.10.15). This article does not contain any studies with human participants that were performed by any of the authors, and no informed consent was applicable.

Establishment of the HINS mouse model

We used KKay male mice, a spontaneous type 2 diabetes animal model, to establish the prediabetic hyperinsulinemia model.²³ The KKay mice were fed a normal diet (KN, n = 8; Beijing Hua Fu Kang Experimental Animal Technology Co., Beijing, China) or a high-fat diet (KHF, n = 8; Beijing Hua Fu Kang Experimental Animal Technology Co.) for 2 weeks. The C57 mice that were fed a normal diet (CN, n = 8) were used as the controls. The normal diet contained 23.8% crude protein, 5.4% crude fat, 3.5% crude fiber, 6.78% total ash, 9% moisture, and 51.2% carbohydrate. The high-fat diet contained 17.98% crude protein, 19.42% crude fat, 4.45% crude fiber, 7.42% total ash, 8.98% moisture, and 41.75% carbohydrate.

Cell culture and siRNA transfection

Human LO2 hepatic cells (a gift from Institute of Endocrinology, Tianjin Medical University) were cultured in RPMI1640 medium that was supplemented with 10% fetal bovine serum, 1% penicillin, and streptomycin in a 37°C incubator that was supplied with 5% CO₂. Control siRNA and siDPP-4 (Invitrogen, Carlsbad, CA, USA) were transfected into 5 × 10⁵ LO2 cells with the Lipofectamine 3000

Transfection Reagent (Life Technologies, Carlsbad, CA, USA) in a 12-well plate, as instructed by the manufacturer’s protocol. After 12 hours, fresh complete medium was changed, and 0.25 mM palmitic acid (Solarbio Co., Beijing, China) was added. Sixteen hours later, the cells were incubated in fresh complete medium for another 12 hours and then harvested for western blot. LO2 cells were also treated with 0.25 mM palmitic acid for 16 hours and subsequently with 30 μmol/L MK0626 (Merck Sharp & Dohme Corp., Whitehouse Station, NJ, USA) for 12 hours. The cells were then harvested for western blot.

Blood glucose and insulin measurement

After the mice had fasted for 14 hours, blood samples were collected from tail veins for blood glucose measurements or for serum insulin measurements after orbital venous sinus bleeding under anesthesia and centrifugation. The blood glucose level was determined using a blood glucose meter (Roche Diagnostics (Shanghai) Co., Shanghai, China). The blood insulin level was determined with a mouse insulin ELISA kit (Millipore, Billerica, MA, USA), in accordance with the manufacturer’s instructions.

Exercise and DPP-4 inhibitor interventions

For the intervention experiments, the mice were fed a normal or high-fat diet for 2 weeks. At 7 weeks of age, the KKay mice were divided randomly into four groups: KKay mice with a normal diet (KN, n = 10), KKay mice with a high-fat diet (KHF, n = 8), KKay mice with the exercise intervention (KE, n = 14), KKay mice with the DPP-4 inhibitor intervention (KD, n = 16). During the 8 weeks of interventions, the KE mice swam 40 minutes every day, and the KD mice were given MK0626 (30 mg/kg body weight) daily by gavage.

MK0626 powder was dissolved in 0.25% sodium carboxymethylcellulose. The KE and KD groups were fed the normal diet during the interventions. C57 mice that were fed the normal diet (CN, $n=10$) were used as the control group. At 15 weeks of age, all the mice were sacrificed. Before sacrifice (exsanguination), the animals were anesthetized using pentobarbital sodium (1%, 50 mg/kg, intraperitoneally). The livers were excised and cut into pieces, which were treated in one of three ways: 1) fixed using 10% formalin and embedded in paraffin; 2) directly embedded in optimal cutting temperature compound (OCT); or 3) frozen in liquid nitrogen.

Hematoxylin and eosin staining

The liver samples were fixed with 10% formalin and embedded in paraffin. Sections (5- μm thick) were cut and deparaffinized with xylene. The sections were hydrated by passing through an ethanol gradient (100%, 95%, 90%, and 80% ethanol). After rinsing with distilled water, the sections were stained in hematoxylin for 3 minutes, washed with tap water for 3 to 5 minutes, differentiated in 0.5% acid alcohol for several minutes, and rinsed in running tap water and Scott's tap water substitute. Subsequently, the sections were rinsed with distilled water and stained with eosin for 3 minutes. The sections were then washed with distilled water, and dehydrated with an ethanol-xylene gradient (75%, 80%, 95%, 100% ethanol first, and then xylene). They were then mounted with Permount (Solarbio Co.) and observed under a microscope.

Oil Red O staining

The liver samples were embedded in OCT and cut into 6- μm sections. The sections were fixed in 4% formalin for 10 minutes and briefly washed with distilled water.

After rinsing with 60% isopropanol for 20 to 30 s, the sections were stained with Oil Red O for 15 minutes. The sections were subsequently rinsed with 60% isopropanol and then water for 1 to 2 minutes and stained with hematoxylin for 1 minute. The sections were then mounted using glycerin jelly and observed under a microscope.

Measurement of liver diacylglycerol

Liver samples (0.2 to 1 g) were washed in ice-cold saline, dried with filter paper, and weighed. Nine volumes of the sample weight of ice-cold saline were added to the samples, and the samples were minced with scissors on ice. The samples were further homogenized with a homogenizer and centrifuged for 15 minutes at 1000 $\times g$ at 4°C. The supernatant was used to measure DAG concentrations using a DAG ELISA kit (EIAB Science Co., Wuhan, China).

Immunohistochemistry

The formalin-fixed and paraffin-embedded sections were deparaffinized and rehydrated. After rinsing with phosphate buffered saline (PBS), the sections were boiled in 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval and incubated in freshly made 3% H_2O_2 to endogenous peroxidase. After washing three times with PBS, the sections were incubated with PKC ϵ (1:50, SC-214, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or IRS-1 tyr (1:50, SC-17196, Santa Cruz Biotechnology) antibodies at 4°C overnight. The next day, the sections were incubated with the corresponding secondary antibodies and then with diaminobenzidine (DAB) for color development. After counterstaining with hematoxylin, the sections were dehydrated, mounted, and photographed. Image-Pro Plus (Media Cybernetics Co., Rockville, MD, USA) was used for the grayscale scanning and analysis.

Protein extraction and western blot

Liver tissue (100 to 500 mg) was homogenized and used for protein extraction. The protein extracts from the cultured cells were also used for the assays that are described below. The protein sample concentrations were determined using a Bradford kit (Beyotime Co., Jiangsu, China). The same amount of protein samples were separated in sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% bovine serum albumin (BSA) and incubated with anti-PKC ϵ (1:1000) or anti-IRS-1 tyr (1:1000), anti-DPP4 (1:1000, ab28340, Abcam, Cambridge, UK), or anti-HSP90 (1:2000, AH732, Beyotime Co.) antibodies. After incubation with the corresponding secondary antibodies, the membranes were detected using an electrochemiluminescence (ECL) kit (Beyotime Co.). The corresponding bands were quantified using grayscale analyses, and the relative expression was calculated by normalizing to β -tubulin (1:2000, AF5012, Beyotime Co.).

Statistical analysis

The data were analyzed using SPSS17.0 (SPSS Inc., Chicago, IL, USA) and presented as the mean \pm standard deviation (SD). Comparisons between two groups were analyzed using the Student's *t*-test, and comparisons among multiple groups were analyzed with an analysis of variance (ANOVA). $P < 0.05$ was considered to be statistically significant.

Results

Establishment of the mouse model of prediabetic hyperinsulinemia

KKay mice were fed a normal (KN, $n = 8$) or a high-fat diet (KHF, $n = 8$) starting at 5

weeks of age. Eight C57 mice that were fed a normal diet (CN) were used as the control mice. The body weight of the mice in the CN, KN, and KHF groups throughout this experiment are shown in Figure 1c. The fasting blood glucose (FBG) levels were comparable among the CN, KN, and KHF mice before they were 7 weeks old (Figure 1a). At week 7, the FBG levels of KN and KHF mice were elevated, but they were not statistically different from the CN mice. However, the FBG levels in the KN and KHF mice increased significantly at week 8 ($P < 0.05$; Figure 1a). The fasting insulin (FINS) levels in KHF mice increased rapidly from 4 to 8 weeks, with a significant difference beginning at week 7 ($P < 0.05$; Figure 1b). The FINS levels of the KHF mice were significantly different compared with the CN mice since week 6. At week 7, the FINS levels of KN mice started to be significantly different compared with that of the CN mice ($P < 0.05$; Figure 1b). Based on the definition for human HINS, we considered the mice to have HINS if their FINS levels were statistically higher than the 95% confidence interval of the FINS for the control C57 mice. The FINS levels of the KHF and CN mice indicated that the KHF mice started to display HINS symptoms at week 7, but they were not diabetic at that time based on the criteria for mouse diabetes (random blood glucose > 16.7 mmol/L).^{20,24}

Exercise and DPP-4 inhibition alleviated hepatic steatosis in mice with HINS

The KKay mice underwent exercise or were administered a DPP-4 inhibitor, MK0626, at week 7. This time point was chosen to start the intervention because the KHF mice already showed HINS symptoms at this age, based on the above experiment. In the intervention groups, the KKay mice were fed a normal diet starting at week 7 to mimic the practical interventions and to meet the clinical principles of diabetes

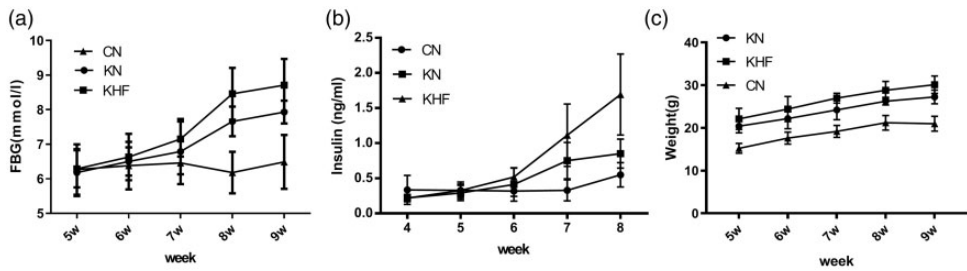


Figure 1. Establishment of the mouse HINS model.

(a) Fasting blood glucose (FBG) in CN (C57, normal diet, $n = 8$), KN (KKay, normal diet, $n = 8$), and KHF (KKay, high-fat diet, $n = 8$) mice at the different ages. At 8 and 9 weeks old, FBG increased significantly in the KN and KHF groups compared with the CN group ($p < 0.05$).

(b) Fasting blood insulin in CN, KN, and KHF mice at different ages. At 8 and 9 weeks old, insulin levels in the KHF group were significantly increased compared with the other two groups ($p < 0.05$).

(c) Body weight of CN, KN, and KHF mice during week 5 to week 9. The body weight of mice in KN and KHF group was significantly higher compared with the CN group from 5 weeks to 9 weeks ($p < 0.05$).

HINS, hyperinsulinemia; CN, C57 mice fed a normal diet; KN, KKay mice fed a normal diet; KHF, KKay mice fed a high-fat diet.

treatments because preventive interventions in the clinic require a normal diet.

The liver is a target organ for insulin, and it plays a critical role in blood glucose homeostasis. Our study aimed to determine if exercise and DPP-4 inhibition exert their protective effects on mice with HINS by improving the mouse liver functions. After the initiation of HINS at the age of 7 weeks, the liver weights of the KN mice were 35.7% heavier compared with the CN mice ($P < 0.05$), indicating that lipid accumulation in the liver is occurs in the KKay mice compared to the C57 mice (Figure 2b). However, the continuous high-fat diet for another 8 weeks dramatically increased the liver weights in the KHF group compared with CN and KN groups ($P < 0.05$, $P < 0.05$, respectively). The liver weights of the KHF group increased by 2.06-fold compared with the CN group at week 15 (Figure 2b). Conversely, the liver weights of KE and KD mice significantly decreased compared with the KN ($P < 0.05$, $P < 0.05$, respectively) and the KHF ($P < 0.01$, $P < 0.01$, respectively) groups.

Histologically, the lobular architecture of the livers was manifested in the CN,

KE, and KD groups (Figure 2a). The hepatocytes aligned radially around the central vein and were tightly packed, having a regular size and shape. No signs of hepatic steatosis were observed. However, indications of hepatic steatosis were present in the KN group, such as hepatocellular ballooning and increased intercellular distance. In the KHF group, more severe hepatic steatosis phenotypes were observed. The hepatocytes contained fat vacuoles, and the nuclei were pushed to the side in the livers in the KHF mice. Perisinusoidal and pericentral fibrosis, portal area expansion, fibrosis spreading into the surrounding parenchyma, and fibrotic scar formation were observed in the livers of KHF mice. These results demonstrated that exercise and DPP-4 inhibition suppressed hepatic steatosis in the mice with HINS.

Exercise and DPP-4 inhibition suppressed the lipid accumulation in the mice with HINS

Next, we stained the liver sections with Oil Red O to examine the TG and lipid

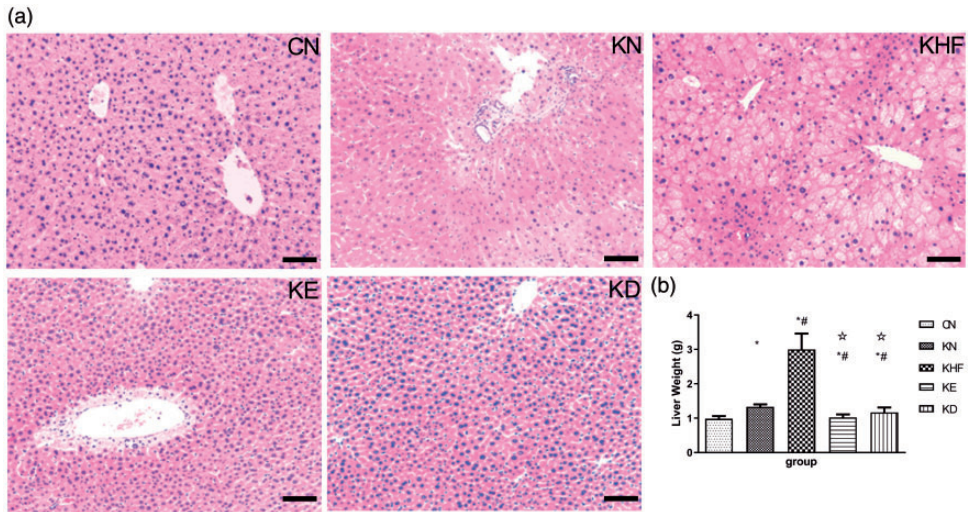


Figure 2. Exercise and DPP-4 inhibition ameliorated hepatic steatosis in the mice with HINS.

(a) Representative images showed the liver histology of CN, KN, KHF, KE, and KD mice.

(b) Exercise and DPP-4 inhibition decreased the liver weight in the KKay mice with HINS compared with the other groups. *, $p < 0.05$ vs. CN group; #, $p < 0.05$ vs. KN group; ☆, $p < 0.01$ vs. KHF group.

CN, C57 mice fed a normal diet ($n = 10$); KN, KKay mice fed a normal diet ($n = 10$); KHF, KKay mice fed a high-fat diet ($n = 8$); KE, KKay mice with the exercise intervention ($n = 14$); KD, KKay mice with the DPP-4 inhibitor intervention ($n = 16$); DPP-4, dipeptidyl peptidase-4; HINS, hyperinsulinemia.

Scale bar: 50 μm .

contents. We observed that the staining intensity significantly increased in the KN group compared with the CN group ($P < 0.01$), and the high-fat diet increased lipid accumulation in the KHF group compared with the CN ($P < 0.01$) and KN ($P < 0.01$) groups (Figure 3a). However, exercise and DPP-4 inhibitor treatment significantly attenuated the staining intensity significantly in the KE and KD groups compared with the KHF groups ($P < 0.01$ for both). Quantification of the staining intensity with grayscale analyses showed exercise and DPP-4 inhibitor treatment significantly reduced the TG and lipid content in the livers compared with the KN or KHF group ($P < 0.01$ for both) (Figure 3b).

DAG is the precursor of TG, and it is an essential mediator of insulin resistance in the liver.⁶ Therefore, we examined the DAG contents in the livers of 15-week-old

CN, KN, KHF, KE, and KD mice. ELISA results showed that the hepatic DAG contents were significantly increased in the KN mice compared with the CN mice ($P < 0.01$), and the high-fat diet led to a further increase of liver DAG in the KKay mice (Figure 3c). Exercise and DPP-4 inhibitor treatment restored the liver DAG levels back to that of the CN mice, suggesting that exercise and the DPP-4 inhibitor intervention suppressed lipid accumulation in the liver.

Exercise and DPP-4 inhibition suppressed PKC ϵ expression to improve insulin resistance

Activation of PKC ϵ by DAG is a critical event that leads to hepatic insulin resistance.²² Because the hepatic DAG levels increased dramatically in the KKay mice,

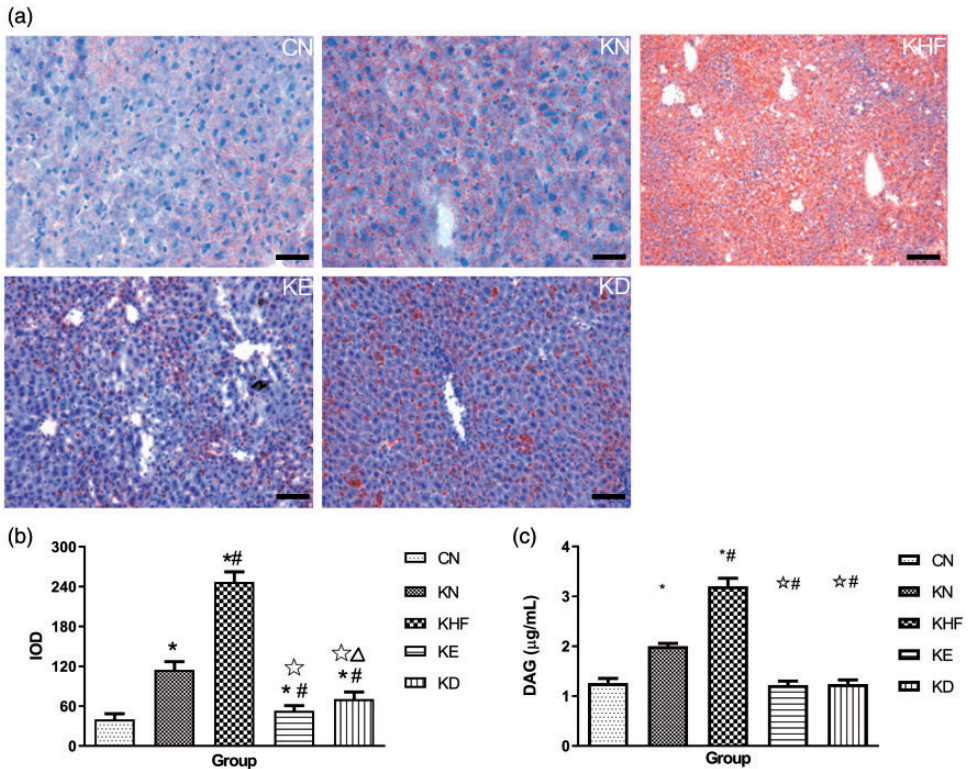


Figure 3. Exercise and DPP-4 inhibition decreased hepatic lipid accumulation in the mice with HINS.

(a) Oil Red O staining showed the hepatic lipid accumulation in CN, KN, KHF, KE, and KD mice.

(b) The quantitative results of the Oil Red O staining showed that exercise and DPP-4 inhibition decreased the liver TG content in the mice with HINS.

(c) ELISA analyses of DAG show that exercise and DPP-4 inhibition decreased the hepatic DAG content in mice with HINS. *, $p < 0.01$ vs. CN group; #, $p < 0.01$ vs. KN group; ☆, $p < 0.01$ vs. KHF group; △, $p < 0.01$ vs. KE group.

CN, C57 mice fed a normal diet ($n = 10$); KN, KKay mice fed a normal diet ($n = 10$); KHF, KKay mice fed a high-fat diet ($n = 8$); KE, KKay mice with the exercise intervention ($n = 14$); KD, KKay mice with the DPP-4 inhibitor intervention ($n = 16$); DPP-4, dipeptidyl peptidase-4; TG, triglycerides; DAG, diacylglycerol; ELISA, enzyme-linked immunosorbent assay; HINS, hyperinsulinemia.

Scale bar: 50 μm .

we next examined whether $\text{PKC}\epsilon$ was activated in the liver of the KKay mice. Immunohistochemistry of $\text{PKC}\epsilon$ showed that the signals were sporadic and light in the livers of the CN group, but were robust and extensive in the livers of the KN and KHF groups (Figure 4a). Compared with the KN and KHF group, the $\text{PKC}\epsilon$ staining signals were significantly reduced in the

livers of KE and KD groups (Figure 4a). The grayscale analyses further confirmed the results that the interventions, especially exercise, suppressed $\text{PKC}\epsilon$ expression in the liver compared with the KHF mice ($P < 0.01$) (Figure 4b). The smaller integrated optical density (IOD) values in the KHF group compared with the KN group might be caused by the increase of fat vacuoles in

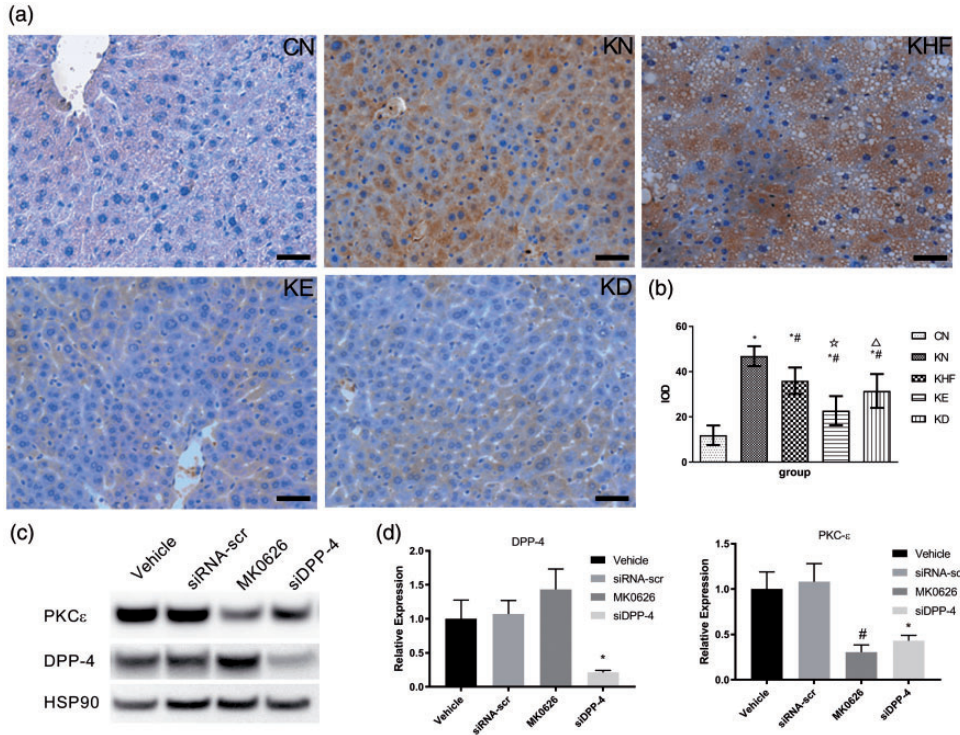


Figure 4. Exercise and DPP-4 inhibition suppressed PKCε expression that was induced in the KKay mice.

(a) Representative IHC images show PKCε expression in the livers of CN, KN, KHF, KE, and KD mice. (b) Grayscale analyses show the quantitative results of PKCε IHC staining. *, p < 0.01 vs. CN group; #, p < 0.01 vs. KN group; ☆, p < 0.01 vs. KHF group; △, p < 0.01 vs. KE group.

(c) Western blot of PKCε and DPP-4 after MK0626 treatment or DPP-4 silencing. HSP90 served as an internal control.

(d) Grayscale analyses showed the quantitative results of PKCε and DPP-4 that were analyzed using western blots. *, p < 0.01 vs. siRNA-scr group; #, p < 0.01 vs. vehicle group. CN, C57 mice fed a normal diet; KN, KKay mice fed a normal diet; KHF, KKay mice fed a high-fat diet; KE, KKay mice with the exercise intervention; KD, KKay mice with the DPP-4 inhibitor intervention; DPP-4, dipeptidyl peptidase-4; PKCε, protein kinase C ε isoform; HSP90, heat shock protein 90; IHC, immunohistochemistry.

Scale bar: 50 μm.

the livers of the KHF group (Figure 4b). The PKCε expression in the livers of CN, KN, KHF, KE, and KD groups was also determined by western blots, which showed similar results as the Immunohistochemistry (IHC) (Figure 5a and b).

To further confirm the regulation of PKCε expression in the liver by DPP-4, we next treated human hepatic LO2 cells

with MK0626 or siRNA that targeted DPP-4 specifically. Our results showed that siDPP-4 significantly decreased DPP-4 expression compared with scrambled siRNA (P < 0.05) (Figure 4a and b). PKCε expression was suppressed in LO2 cells that were transfected with siDPP-4 (Figure 4c and d). Because MK0626 only inhibited DPP-4 activity, and not its expression, MK0626 treatment did not decrease

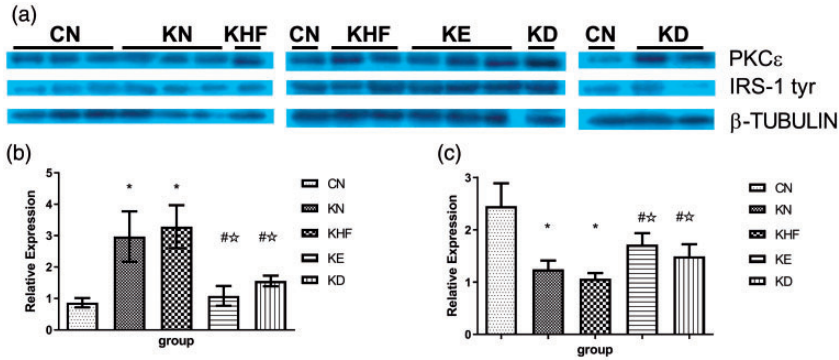


Figure 5. Western blot confirmed that exercise and DPP-4 inhibition suppressed PKC ϵ expression and activated insulin signaling in mice with HINS.

(a) Western blots of PKC ϵ and IRS-1 tyrosine phosphorylation in livers from CN, KN, KHF, KE, and KD mice. β -Tubulin served as an internal control.

(b) Grayscale analyses showed the quantitative results of the PKC ϵ western blots. *, $p < 0.01$ vs. CN group; #, $p < 0.01$ vs. KN group; ☆, $p < 0.01$ vs. KHF group.

(c) Grayscale analyses showed the quantitative IRS-1 tyrosine phosphorylation results on western blots. *, $p < 0.05$ vs. CN group; #, $p < 0.05$ vs. KN group; ☆, $p < 0.05$ vs. KHF group.

CN, C57 mice fed a normal diet; KN, KKay mice fed a normal diet; KHF, KKay mice fed a high-fat diet; KE, KKay mice with the exercise intervention; KD, KKay mice with the DPP-4 inhibitor intervention; DPP-4, dipeptidyl peptidase-4; PKC ϵ , protein kinase C ϵ isoform.

DPP-4 expression. However, PKC ϵ expression was markedly suppressed in MK0626 treated cells compared with the vehicle-treated cells ($P < 0.01$; Figure 4c and d). These results further demonstrated that DPP-4 inhibition caused the reduction of PKC ϵ expression.

PKC ϵ prevents tyrosine phosphorylation of IRS-1 and interferes with the transduction of insulin signals.⁶ Therefore, we also used IHC and western blots to determine the IRS tyrosine phosphorylation levels in the liver (Figure 5a and c, Figure 6a and b). The grayscale analysis of IHC graphs showed that, compared with the CN group, tyrosine phosphorylation of IRS-1 was significantly reduced in the livers of KN and KHF mice ($P < 0.01$ for both; Figure 6a and b). Exercise and DPP-4 inhibition partially restored the tyrosine phosphorylation in the livers. Western blots confirmed the IHC results (Figure 5a and c). These results suggested that exercise and DPP-4 inhibition might

improve hepatic insulin resistance by suppressing PKC ϵ expression.

Discussion

Exercise training, dietary changes, metabolic medication, and bariatric surgery can efficiently improve HINS,⁵ which is an early indicator of T2DM. Thus, HINS interventions may be an effective means to delay and prevent the onset of T2DM. In this study, we used a mouse model of spontaneously developed HINS and showed that a DPP-4 inhibitor, MK0626, had a similar effect as exercise in ameliorating the hepatic steatosis and reducing the liver TG and DAG content. Hepatic DAG-induced PKC ϵ expression was remarkably suppressed by DPP-4 inhibition both *in vivo* and *in vitro*, suggesting that suppressing PKC ϵ expression in the liver is a molecular mechanism that mediates the protective effect of DPP-4 inhibition. Thus, insulin signaling, which was determined by the

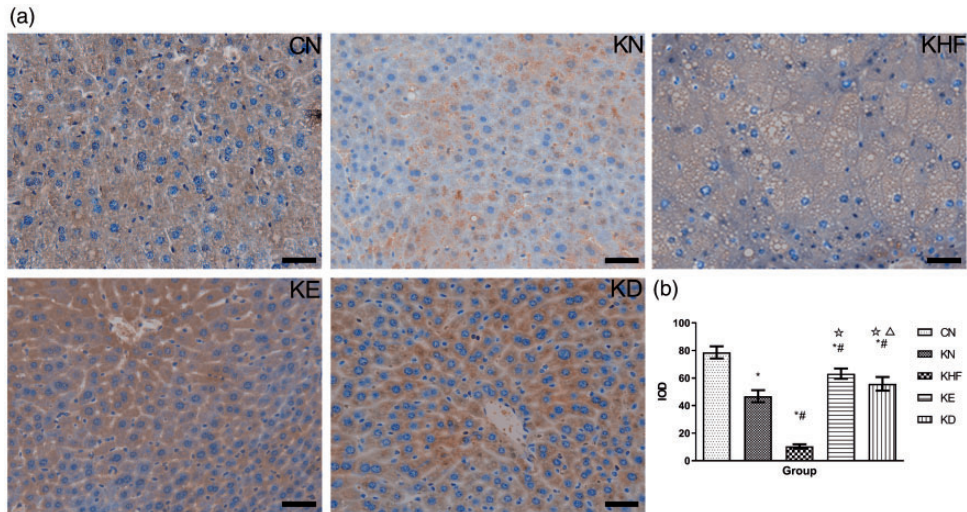


Figure 6. Exercise and DPP-4 inhibition activated insulin signaling in the mice with HINS.

(a) Representative IHC images show IRS-1 tyrosine phosphorylation in the livers of CN, KN, KHF, KE, and KD mice.

(b) Grayscale analyses show the quantitative results of the IRS-1 tyrosine phosphorylation IHC staining.

* $p < 0.01$ vs. CN group; #, $p < 0.01$ vs. KN group; ☆, $p < 0.01$ vs. KHF group; △, $p < 0.01$ vs. KE group.

CN, C57 mice fed a normal diet; KN, KKay mice fed a normal diet; KHF, KKay mice fed a high-fat diet; KE, KKay mice with the exercise intervention; KD, KKay mice with the DPP-4 inhibitor intervention; siRNA-scr, scramble siRNA; DPP-4, dipeptidyl peptidase-4; siDPP-4, siRNA targeting DPP-4; siRNA, small interfering RNA.

Scale bar: 50 μ m.

tyrosine phosphorylation of IRS-1, was suppressed in the livers of KKay mice and was restored after exercise or MK0626 intervention.

HINS is associated with the incidence of NAFLD.⁴ NAFLD is a primary cause of hepatic insulin resistance in humans.⁸ KKay mice develop hepatic steatosis spontaneously, and a high-fat diet induces even more severe lipid accumulation in the liver, showing that HINS in this model will progress into NAFLD and eventually T2DM, especially under the treatment of the high-fat diet (Figures 2 and 3). However, early interventions with both exercise and DPP-4 inhibition ameliorated hepatic steatosis (Figures 2 and 3) and improved insulin resistance (Figures 5 and 6) in the KKay mice. These results suggested that the protective effects of DPP-4 inhibition in our model are similar to exercise and are at

least partially attributed to the reduction of hepatic steatosis.

Increased intrahepatic TG, which is a hallmark of NAFLD, was associated with hepatic insulin resistance.²² However, it is becoming increasingly accepted that DAG, the precursor of TG, is the dominant player in the lipid-induced hepatic insulin resistance. Intrahepatic DAG activates PKC ϵ and results in the impairment of insulin receptor signaling transduction and insulin resistance. Suppressing PKC ϵ protected rats from hepatic insulin resistance that is induced by hepatic steatosis.⁶ Thus, hepatic DAG and PKC ϵ activation are associated with hepatic insulin resistance in humans⁸ and mice.²¹ Our study showed that similar to exercise, DPP-4 inhibition decreased the intrahepatic DAG levels and suppressed PKC ϵ expression in the liver (Figures 3c, 4, and 6). Silencing DPP-4 or MK0626

treatment in human hepatic LO2 cells further confirmed the link between DPP-4 inhibition and PKC ϵ expression. This novel molecular mechanism at least partially explains how these interventions protected the HINS mice from developing T2DM.

Hepatic insulin exerts its functions in energy homeostasis through binding to insulin receptors on the membranes of hepatic cells.⁶ The activation of insulin receptor tyrosine kinase leads to the phosphorylation of IRS-1 and IRS-2 and triggers PI3K and AKT cascades. By examining IRS-1 tyrosine phosphorylation, we showed that insulin signaling was impaired in KN mice, especially in the KHF mice, which shows the extent of hepatic insulin resistance in the KKay mice (Figures 5 and 6). However, exercise and DPP-4 inhibition partially restored IRS-1 tyrosine phosphorylation, suggesting that there is better insulin signaling in the KE and KD mice compared with the KN and KHF mice (Figures 5 and 6). This result is consistent with our previous report, which showed that exercise and DPP-4 inhibition protect HINS mice from developing T2DM.²⁰

Data supporting the impact of exercise on lipid-induced hepatic insulin resistance are limited.⁶ The recent discovery of the essential role of DAG in insulin resistance links the protective effects of exercise with insulin resistance. Our data (Figure 3c) and a previous report supported that exercise reduced deleterious DAG accumulation in the liver.²⁵ We first demonstrated here that exercise-induced DAG reduction led to a decrease of PKC ϵ expression in the liver (Figures 4 and 6), and this activation impairs insulin sensitivity. Thus, we speculated that reduced hepatic DAG content contributed significantly to the protective effects of exercise in our model. Our studies showed that exercise had a more profound impact on hepatic steatosis (Figures 2b and 3) and insulin resistance (Figures 4, 5, and 6)

compared with DPP-4 inhibition. It has also been shown that exercise is a better intervention for T2DM compared with metformin in a rat model.²⁶ These results highlight the importance of a lifestyle change.

Additionally, some patients cannot adhere to the new lifestyle or they refuse to exercise. Pharmacological interventions have become a hot topic of metabolic research. Although DPP-4 inhibitors have been widely used for blood glucose management for diabetic patients, whether they can be used as a protective drug in HINS or prediabetic patients remains unclear. Several studies suggested that some DPP-4 inhibitors might be used as protective drugs in prediabetic patients primarily through ameliorating hepatic steatosis. The DPP-4 inhibitor MK0626 decreased hepatic TG and DAG accumulation and reduced hepatic steatosis and insulin resistance that were caused by a high-fat/high-fructose diet.²⁷ Another DPP-4 inhibitor, teneligliptin, moderately reduced hepatic steatosis and improved insulin resistance in a NAFLD mouse model.²⁸ However, DPP-4 inhibitor sitagliptin did not improve hepatic steatosis that was assessed by noninvasive imaging techniques in prediabetic patients with NAFLD.²⁹ A variation in effects may arise from the specificity of different DPP-4 inhibitors, the subjects who were chosen in the study, and the methods used for hepatic steatosis evaluation. The activation of PPAR- α in the liver of DPP-4 ablated mice³⁰ explains the reduced hepatic accumulation of TG and DAG and the increased phosphorylation of IRS-1 in the liver that is induced by DPP-4 inhibition.^{31,32} However, the link between the DPP-4 inhibition-induced hepatic steatosis amelioration and improvement in insulin sensitivity is still weak. Here, using *in vivo* and *in vitro* experiments, we showed that the reduction of intrahepatic DAG accumulation induced by DPP-4 inhibition suppressed PKC ϵ expression, which

restored IRS-1 tyrosine phosphorylation (Figures 4, 5, and 6). This novel discovery provides mechanistic insight into how DPP-4 inhibition prevents the onset of T2DM in HINS and prediabetic patients.

Conclusions

We showed that early intervention by DPP-4 inhibition is similar to exercise and effectively prevents HINS from progressing into T2DM. Similar to exercise, DPP-4 inhibition ameliorates hepatic steatosis and decreases the DAG level, which in turn inhibits PKC ϵ expression and improves insulin signaling. This study illustrates how DPP-4 inhibition suppresses PKC ϵ expression in the liver to prevent HINS from progressing into T2DM using a HINS mouse model, which provides instructive information for the early prevention of T2DM in the clinic, and it may extend to humans in the future.

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Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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