

Fenofibrate Attenuates Renal Tubular Cell Apoptosis by Up-Regulating MCAD in Diabetic Kidney Disease

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Background: Diabetic kidney disease (DKD) is a major diabetic microvascular complication. Fatty acid-induced lipotoxicity and apoptosis were associated with the exacerbation of DKD. However, the association of lipotoxicity with renal tubular apoptosis and the effects of fenofibrate on DKD are not fully understood.

Methods: Eight-week-old db/db mice were given fenofibrate or saline by gavage for 8 weeks. Human kidney proximal tubular epithelial (HK2) cells stimulated with palmitic acid (PA) and high glucose (HG) were used as a model of lipid metabolism disorders. Apoptosis was assessed with or without fenofibrate. The AMP-activated protein kinase (AMPK) activator 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and AMPK inhibitor Compound C were used to determine the involvement of AMPK and Medium-chain acyl-CoA dehydrogenase (MCAD) in the regulation of lipid accumulation by fenofibrate. MCAD silencing was achieved by small interfering RNA (siRNA) transfection.

Results: Fenofibrate reduced triglyceride (TG) content and lipid accumulation in DKD. Importantly, renal function and tubular cell apoptosis were significantly improved by fenofibrate. Fenofibrate reduced apoptosis, accompanied by increased activation of the AMPK/FOXO2/MCAD pathway. MCAD silencing resulted in apoptosis and lipid accumulation despite fenofibrate treatment.

Conclusion: Fenofibrate improves lipid accumulation and apoptosis through the AMPK/FOXO2/MCAD pathway. MCAD may be a potential therapeutic target of DKD, and the use of fenofibrate as a treatment for DKD warrants further study.

Keywords: fenofibrate, diabetic kidney disease, renal tubular cell, apoptosis, lipid accumulation, MCAD

Introduction

Diabetic kidney disease (DKD) is a typical complication of diabetes mellitus, associated with higher mortality and morbidity, and is a major cause of end-stage renal disease (ESRD).¹ DKD is clinically characterized by albuminuria and a progressive decline in kidney function.² Although glomerular pathology is thought to be the primary target of diabetic injury, it has been well documented that tubulointerstitial injury plays an important role in the progression of DKD.³ Renal tubular cell apoptosis is clearly recognized as a key pathogenesis of chronic kidney injury and is associated with the progression of renal fibrosis.⁴ Previous work has shown that high glucose (HG) conditions induce apoptosis of renal tubular epithelial cells in vitro and in vivo.^{5,6} Abnormal lipid flow and lipid deposition have been identified as major risk factors for the progression of diabetes. In recent years, it has been increasingly recognized that lipid accumulation is also largely involved in the development of DKD.⁷ Lipid accumulation in the kidney causes lipotoxicity, which may lead to kidney damage and further transform into kidney fibrosis.^{5,6}

Medium-chain acyl-CoA dehydrogenase (MCAD) is a mitochondrial enzyme involved in the β -oxidation of medium-chain fatty acids (MCFA).⁸ MCAD deficiency leads to lipid deposition in several tissues, but its role in DKD remains

unclear.⁹ AMP-activated protein kinase (AMPK), a serine/threonine kinase, acts as an energy sensor in the homeostasis of carbohydrate and lipid metabolism.^{10,11} Downregulation of AMPK and tubular damage were observed in DKD.¹² Studies have shown that AMPK can inhibit forkhead box A2 (FOXA2) nuclear excretion and reduce lipid deposition.¹⁰ FOXA2 is a transcription factor belonging to the forkhead box family that plays a regulatory role in the maintenance of glucose and lipid homeostasis.^{13,14} FOXA2 promotes MCAD transcription by binding upstream of the MCAD promoter.¹⁵ FOXA2 activates the transcriptional program of lipid metabolism under normal conditions. In insulin-resistant mice, plasma insulin inhibits FOXA2 by nuclear exclusion and FOXA2 was inactive and permanently located in the cytoplasm of hepatocytes.^{11,13,16}

Fenofibrate is widely used as an effective drug for the treatment of hypertriglyceridemia.¹⁷ The DAIS study indicated that fenofibrate reduced microalbuminuria in diabetic patients.¹⁸ Previous studies have shown that fenofibrate could improve diabetic complications, which is attributed to its anti-oxidant and anti-inflammatory properties. However, the mechanism by which fenofibrate to improve the progression of DKD is not fully understood.^{19,20} In this study, we investigated the effect of fenofibrate on renal tubules. We explored the underlying mechanisms and examined the roles of the AMPK/FOXA2/MCAD pathway and apoptosis, which may be key players in the regulation of renal tubular injury. We demonstrated that fenofibrate could ameliorate renal lipotoxicity-induced apoptosis and that this effect is mediated through the AMPK-FOXA2-MCAD pathway.

Materials and Methods

Animals

The db/db mouse, which has genetic defects in the leptin receptor, is a well-established model for type 2 diabetes. Six-week-old male db/db mice and their control m/m mice used for animal experiments were purchased from GemPharmatech Ltd. in Jiangsu, China. Mice were maintained at room temperature (20–24°C) and fed a standard chow diet ad libitum in environmentally controlled animal facilities at Tianjin Institute of Endocrinology. After 2 weeks of adaptation, db/db mice were randomly divided into two groups: db/db mice (vehicle control, n=6) and db/db mice received fenofibrate (Recipharmfontaine, France, 100 mg/kg/day, n=6) by intragastric gavage daily for 8 weeks. As a control group, m/m mice (n = 6) were treated with matching volumes of saline for the same period of time. Blood glucose (sampled from the tail) and body weight were measured twice weekly. All mice were sacrificed at 16 weeks of age. After collecting blood samples, the left kidney weight (KW) was measured, and kidney tissues were collected for further experiments. All animal experiments were approved by the Ethics Committee of Tianjin Medical University Chu Hsien-I Memorial Hospital (Tianjin, China) (DXBYY-IACUC-2023002) and followed the guidelines of the Animal Welfare Council of China.

Assessment of Physiological Features

24-hour urine samples from mice were collected using metabolic cages. Urinary albumin (uALB) and β 2-microglobulin (β 2-MG) concentrations were measured using the respective ELISA kits (Jianglai Biotechnology, China). Urinary albumin excretion was calculated as the total amount in 24 h. Serum and kidney triglyceride (TG) levels were assessed using TG assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

Histological Analysis

Paraffin-embedded kidney tissue sections of 4 mm thickness were prepared for histological analysis. The sections were stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS), and Masson trichrome (Masson) staining kits according to the manufacturer's protocol. The pathological changes in kidney tissue were viewed under a light microscope with a digital camera (Olympus, Japan). To evaluate the degree of damage to renal tubules in mice, 400 $\times$ visual fields were randomly selected from each section, and the number of healthy renal tubules was manually counted using the Adobe Photoshop counting tool. Tubules were defined as healthy when the dimension, structure, relative nucleus-cytoplasm configuration, brush border, and basal membrane integrity were similar to those of healthy tubules. The area of renal fibrosis

stained with aniline blue dye was extracted using ImageJ. The fibrotic area was measured, which was divided by the interstitial area to obtain the renal fibrosis area ratio, the mean value of which was used for statistical analysis.

Immunohistochemistry

Paraffin-embedded sections were deparaffinized, rehydrated, blocked, and incubated with primary antibody, followed by incubation with HRP-labeled secondary antibody, staining with diaminobenzidine substrate, and counterstaining with hematoxylin. Immunoreactivity was assessed by optical microscopy.

Determination of Triglyceride (TG) Content

A TG assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) was used to determine triglyceride content. The assay was conducted according to the manufacturer's instructions. A standard curve was produced with TG standard solution. Three biological replicates were established and the absorption at 510 nm was determined.

Oil Red O Staining

Frozen kidney sections and cells were fixed in 4% paraformaldehyde, washed with PBS for 10 min, stained with Oil Red O solution (0.5% isopropyl alcohol, diluted 3:2 with ddH₂O) for 15–20 min at room temperature, washed three times with PBS, and finally counterstained with hematoxylin. Images were taken by Olympus I3-TPC microscope. To quantify Oil Red O staining, intracellular lipids were extracted with isopropanol and shaken for 10 min at room temperature, and absorbance was measured at 520 nm on a monochromator microplate reader (Bio Tek).

Cell Culture and Treatments

Human proximal tubular epithelial cells (HK2 cells) were acquired from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and maintained in DMEM/F12 medium (Gibco, United States) containing 10% fetal bovine serum (Gibco, United States) in a humidified incubator at 37°C and 5% CO₂. When HK2 cells reached 60–70% confluence in six-well plates, HK2 cells were treated with palmitic acid (PA) and high glucose (HG). Mannitol was used to control osmolality. The applied concentration of fenofibrate was 50 µmol/L according to the results of the cell viability assay performed with Cell Counting Kit-8 (CCK8). To determine the effect of AMPK, HK2 cells were induced with 0.1 mmol/L PA and 25 mmol/L glucose for 24 h, and then treated with or without fenofibrate (50 µmol/L) and with or without 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) (0.5 mmol/L) or Compound C (20 µmol/L) treatment. All in vitro experiments were repeated at least 3 times with 3 triplicates.

TUNEL Assay

The TUNEL assay kit (Beyotime Biotechnology, China) was used to detect apoptosis. Briefly, kidney sections were dewaxed and then incubated with proteinase K. Kidney sections or cells were stained with a TUNEL reaction mixture consisting of TdT and fluorescein-labeled dUTP solution. Finally, the nuclei were stained with DAPI. Images were captured by a fluorescence microscope equipped with a digital camera (Olympus, Japan). The apoptotic rate was quantified with the TUNEL positive rate.

Isolation of Nuclear and Cytoplasmic Protein

The nuclear and cytoplasmic fractions were separated with a Nuclear and Cytoplasmic Protein Extraction Kit (P0028, Beyotime, China). The procedure was carried out according to the manufacturer's protocol. Protein expression in the samples was analyzed by Western blot.

Western Blot Analysis

Proteins from kidney tissues or HK2 cells were extracted with RIPA buffer (Solarbio, China) containing protease inhibitor cocktail, and protein concentrations were determined with a BCA protein assay kit (Thermo, USA). Then, proteins were separated by SDS/PAGE, transferred to nitrocellulose filter membranes, blocked with 5% milk for 1 h at room temperature, and then the membranes were incubated with the following primary antibodies overnight at 4°C: Bax

(1:1000, Proteintech, No. 50599-2-IG), Bcl-2 (1:2000, CST, 4223s), Caspase 3/p17/p19 (1:1000, Proteintech, No.19677-1-AP), p-AMPK (1:2000, Abcam, ab23875), AMPK (1:2000, Abcam, ab80039), MCAD (1:10,000, Abcam, ab92461), FOXA2 (1:1000, Proteintech, No.22474-1-AP), H3 (1:10,000, Abcam, ab1791), GAPDH (1:10,000, Bioworld, AP0066). After washing with TBST, the membranes were incubated with secondary antibodies for 1 hour and then visualized with an enhanced chemiluminescence system. Band intensities were analyzed using ImageJ software and normalized to the expression of GAPDH.

siRNA Transfection

Specific MCAD siRNA (5'-GCUCUGAUGUAGCUGGUAUTT -3' and 5'-AUACCAGCUACAUCAGAGCTT-3') and negative control siRNA were obtained from GenePharma. MCAD siRNA was transfected with Lipofectamine 2000 (Invitrogen, USA) for 6 h according to the manufacturer's protocol. After transfection for 6h, the cells were treated with PA, HG and fenofibrate for 24 h.

Statistical Analysis

GraphPad Prism 7.0 software was used for statistical analyses. All values are presented as the mean $\pm$ SE. QQ plots were used to assess normality of data. One-way ANOVA with Tukey's test was utilized to determine significant differences among multiple groups, and unpaired Student's t-tests were used to compare the differences between two groups. Statistical significance was considered at a value of $p < 0.05$.

Results

Fenofibrate Improves Renal Function and Alleviates Renal Pathological Damage in db/db Mice

The BKS-db/db mice is a valid model for T2DM and has been shown to develop diabetic kidney injury. After 8 weeks of fenofibrate treatment, db/db mice showed a decrease in body weight, blood glucose and serum triglycerides, but did not affect kidney weight (Figure 1A–D). Notably, fenofibrate significantly reduced urinary albumin (uALB) excretion and β 2-microglobulin (β 2-MG) in db/db mice, indicating improved renal function (Figure 1E and F). Histologically, fenofibrate treatment significantly improved renal tubule health status and tubulointerstitial injury characterized by renal tubular cells derangement, death and tubular dilatation (Figure 1G and H). In addition, the accumulation of glycogen shown by PAS staining and tubulointerstitial fibrosis shown by Masson staining were increased in the kidneys of db/db mice relative to m/m mice, which were significantly alleviated after fenofibrate treatment (Figure 1G and I).

Fenofibrate Ameliorates Lipid Accumulation and Apoptosis and Regulates AMPK/FOXA2/MCAD Signaling in db/db Mice

We next explored the effect of fenofibrate on lipid deposition and apoptosis in the kidney. Oil red O staining showed that the kidneys of db/db mice showed lipid accumulation, which was significantly alleviated by fenofibrate (Figure 2A). Consistently, kidney triglyceride (TG) levels were also decreased (Figure 2B). Excessive lipid accumulation may induce lipotoxicity, leading to cell injury and apoptosis.²¹ To determine whether apoptosis was increased in db/db mice and whether this change was restored by fenofibrate, apoptosis was assessed with the apoptosis-associated proteins, Bax, Bcl-2 and Cleaved caspase 3. Western blot analysis showed that diabetes significantly exacerbated the level of apoptosis, whereas apoptosis was restored in the kidneys of db/db mice after fenofibrate administration (Figure 2C). In addition, TUNEL assay was performed and the results showed that fenofibrate reduced apoptosis in the kidneys of db/db mice (Figure 2D and E). MCAD is one of the significant enzymes involved in the β -oxidation of fatty acids. MCAD deficiency affects the β -oxidation of fatty acids and leads to lipid deposition in several organs, but little is known about its importance in DKD. It has been shown that fenofibrate positively regulates fatty acid β -oxidation by upregulating the expression levels of lipolytic enzymes. In our research, we found that MCAD was reduced in db/db mice, and it was restored by fenofibrate treatment (Figure 2F). Given that MCAD has been reported to be regulated by the AMPK/FOXA2 signaling pathway, we evaluated whether the AMPK/FOXA2/MCAD signaling pathway is altered in DKD. Western blot

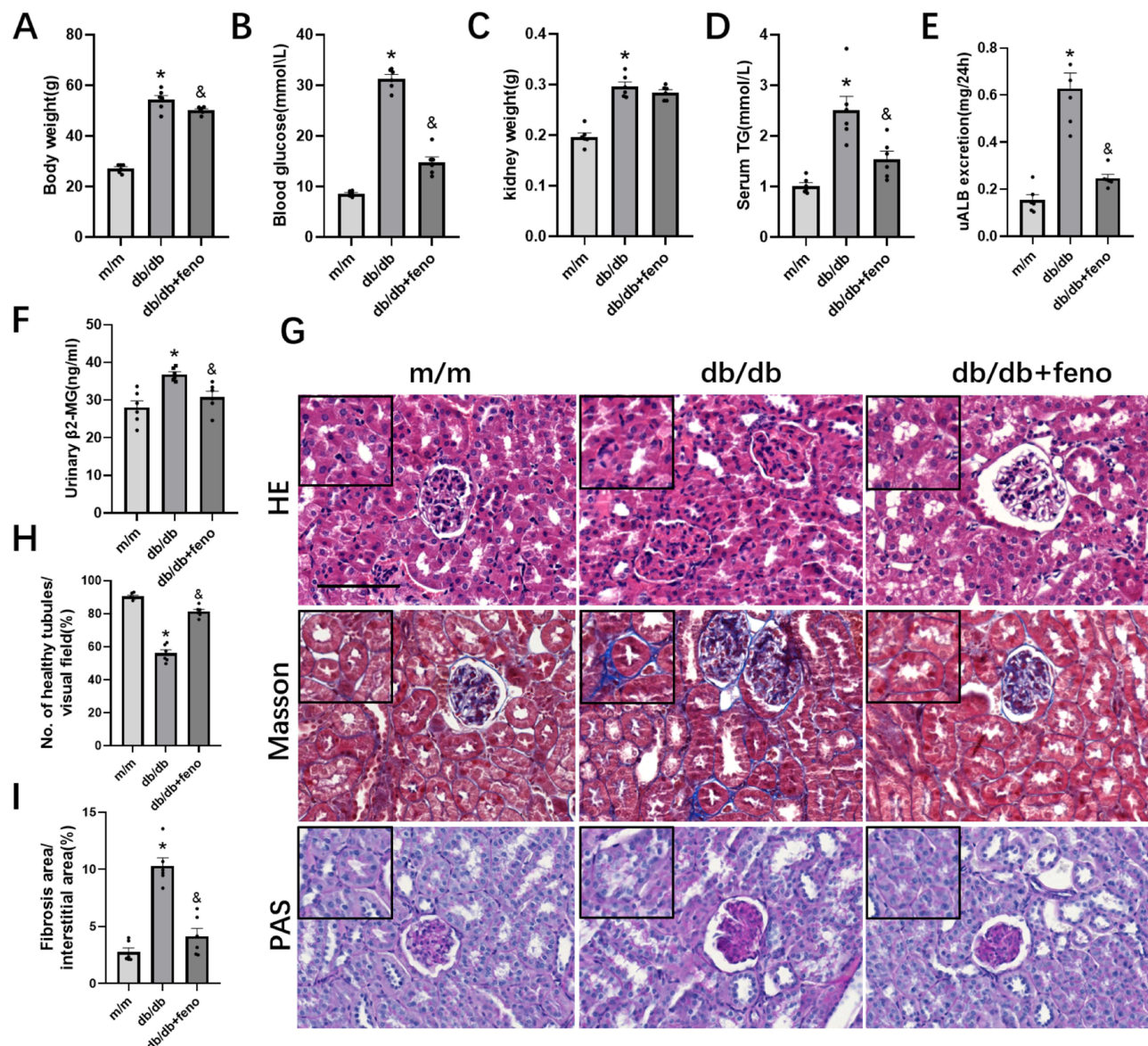


Figure 1 Fenofibrate improves renal function and alleviates renal pathological damage in db/db mice. (A) Body weight, n=6. (B) Fasting blood glucose, n=6. (C) Kidney weight, n=6. (D) Serum triglyceride (TG) concentrations, n=6. (E) 24h-urinary albumin (uALB) excretion, n=6. (F) Urinary β2-microglobulin (β2-MG), n=6. (G) Representative images of kidney sections stained with HE, PAS and Masson's trichrome. Original magnification = 400×. Scale bar = 100 μm. (H) Number of healthy tubules, on the basis of HE staining, n=6. (I) The ratio of fibrosis area to renal interstitial area in each group was calculated, on the basis of Masson's trichrome staining, n=6. Eight-week-old male mice were gavaged with saline or fenofibrate for 8 weeks. All data are presented as the means ± SEs. feno, fenofibrate. *P < 0.05 vs. the m/m mice group; #P < 0.05 vs. the db/db mice group.

showed decreased levels of phosphorylated AMPK and nuclear FOXA2 and increased cytoplasm FOXA2, which can be restored by fenofibrate (Figure 2F and G). Furthermore, immunohistochemical analysis revealed that MCAD was expressed mostly in the renal tubules (Figure 2H). Therefore, fenofibrate may reduce lipid deposition and apoptosis in renal tubules through activation of the AMPK/FOXA2/MCAD signaling pathway, thereby attenuating DKD.

Fenofibrate Reduces Lipid Accumulation and Apoptosis in vitro

To confirm the effect of fenofibrate on lipid accumulation and apoptosis in vitro, we used human kidney proximal tubular epithelial (HK2) cells as a cellular model, since proximal renal tubular epithelial cells depend mainly on fatty acid oxidation (FAO) as their energy source.²² After experimenting with different concentrations of PA (0, 0.025, 0.05, 0.1, 0.2 mmol/L) for 24h, treatment with 0.1 mmol/L PA and 25 mmol/L glucose was determined to be the optimal condition (Figure 3A). We then treated HK2 cells with 50 μmol/L fenofibrate to investigate the effects of fenofibrate, which did not

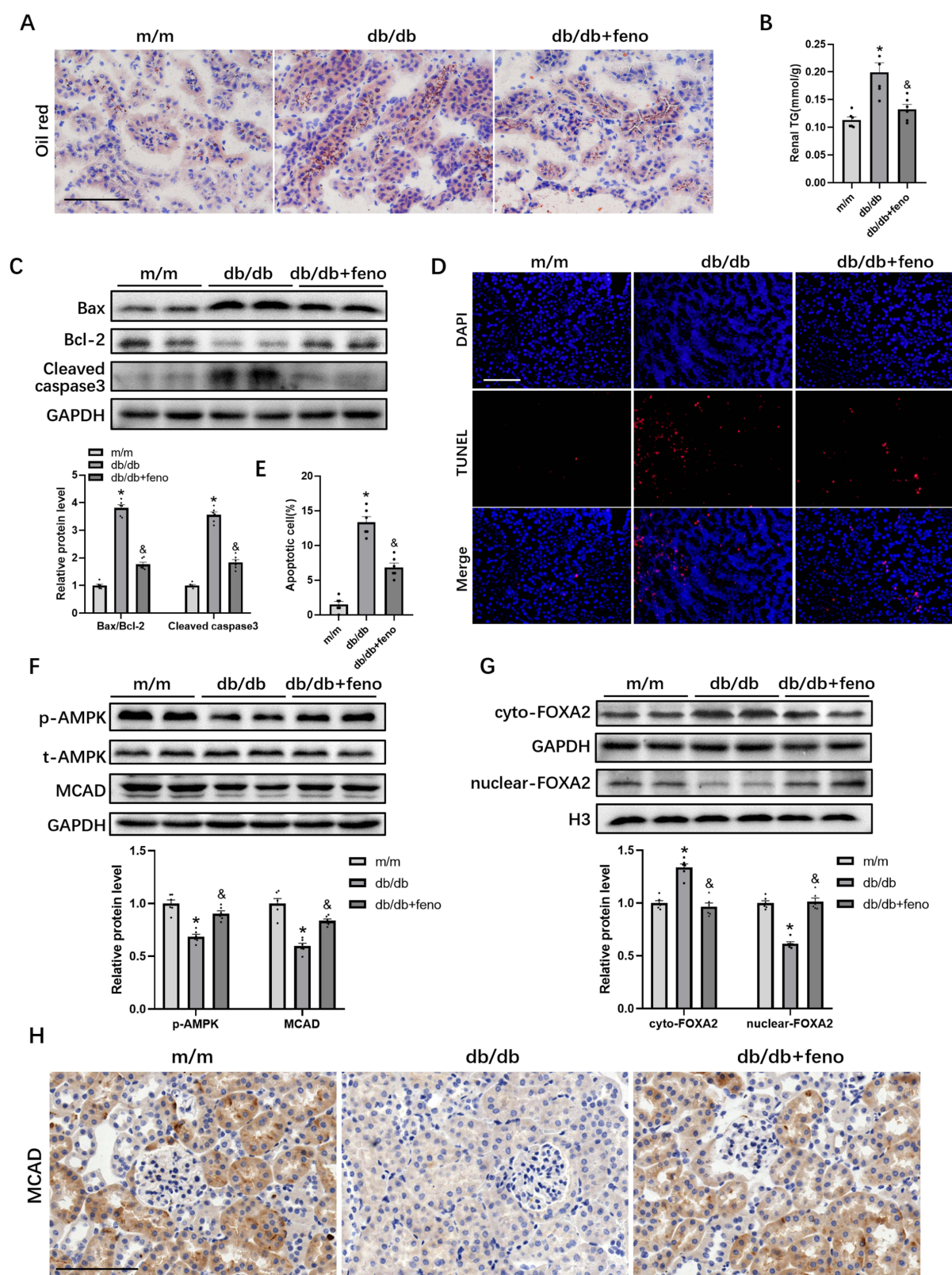


Figure 2 Fenofibrate ameliorates lipid accumulation and apoptosis and regulates AMPK/FOXO2/MCAD signaling in db/db mice. **(A)** Representative images of Oil Red O staining. Scale bar = 100 μ m. **(B)** Renal triglyceride (TG), n=6. **(C)** Representative blots and a quantitation graph of Bax, Bcl-2 and Cleaved caspase 3 protein expression in the kidney, n=6. **(D)** Representative images of TUNEL staining. Apoptotic cells are visualized as red and nuclei are stained with DAPI (blue), Scale bar = 100 μ m. **(E)** Quantitative analysis for the number of TUNEL-positive cells. **(F and G)** Representative blots and a quantitation graph of p-AMPK, t-AMPK, MCAD, cyto-FOXO2, nuclear-FOXO2 protein expression in the kidney, n=6. **(H)** Representative micrographs showing kidney MCAD immunohistochemistry (IHC) staining, scale bar = 100 μ m. Eight-week-old male mice were gavaged with saline or fenofibrate for 8 weeks. All data are presented as the means $\pm$ SEs. feno, fenofibrate. *P < 0.05 vs. the m/m mice group; &P < 0.05 vs. the db/db mice group.

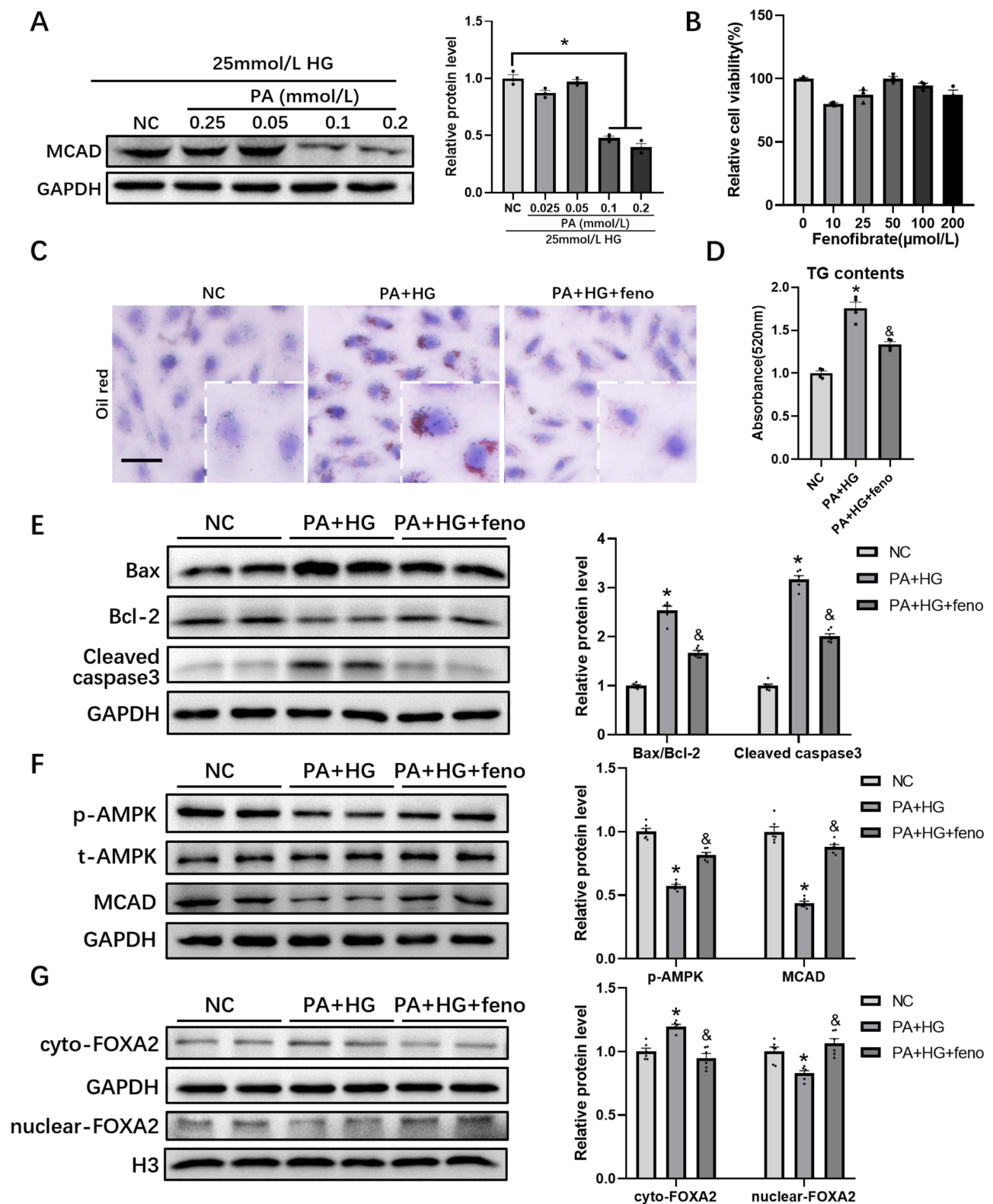


Figure 3 Fenofibrate reduces lipid accumulation and apoptosis in HK2 cells. **(A)** Representative blots and a quantitation graph of MCAD protein expression in HK2 cells, HK2 cells were treated with different concentrations of (0, 0.25, 0.05, 0.1, 0.2 mmol/L) PA and 25 mmol/L glucose for 24h, n=3. **(B)** Cell viability measured by cell counting kit-8 (CCK8) after treatment with different doses of fenofibrate, n=3. **(C)** Representative images of Oil Red O staining and **(D)** Quantitative analysis of triglyceride (TG), n=4. Scale bar = 50 μm. **(E)** Representative blots and a quantitation graph of Bax, Bcl-2 and Cleaved caspase 3 protein expression in HK2 cells, n=6. **(F and G)** Representative blots and a quantitation graph of p-AMPK, t-AMPK, MCAD, cyto-FOXA2, nuclear-FOXA2 protein expression in the HK2 cells, n=6. HK2 cells were stimulated with 0.1 mmol/L PA and 25 mmol/L glucose in the presence or absence of pretreatment with fenofibrate (50 μmol/L). All data are presented as the means ± SEs. *P < 0.05 vs. the NC group; [§]P < 0.05 vs. the PA + HG group. **Abbreviations:** NC, negative control group; HG, high glucose; PA, palmitic acid; feno, fenofibrate; cyto, cytoplasmic; HK2 cells, human kidney proximal tubular epithelial cells.

affect cell survival (Figure 3B). Treatment with fenofibrate significantly reduced lipid accumulation in HK2 cells, as evidenced by Oil Red O staining (Figure 3C) and TG levels (Figure 3D). We next evaluated cell apoptosis in the cell model. Western blot showed an increased levels of Bax/Bcl-2 ratio and Cleaved caspase 3 in cells under PA- and high glucose (HG)-induced steatosis, while fenofibrate treatment reversed this effect (Figure 3E). Meanwhile, PA- and HG-induced a decrease in the protein levels of phosphorylated AMPK, nuclear FOXA2 and MCAD in HK2 cells, but fenofibrate significantly upregulated the expression of those proteins (Figure 3F and G). These data suggest that fenofibrate activated the AMPK/FOXA2/MCAD signaling pathway, reduced lipid accumulation, and ameliorated apoptosis in HK2 cells.

MCAD is Required for Fenofibrate to Reduce Lipid Accumulation and Apoptosis

To investigate the potential role of MCAD in lipid accumulation and apoptosis, siRNA targeting MCAD was transfected into HK2 cells. The preventive effect of fenofibrate on PA- and HG-induced lipid accumulation was abolished when MCAD was depleted. Inhibition of MCAD appeared to increase the number and relative size of lipid droplets in HK2 cells (Figure 4A). The importance of MCAD for lipid accumulation was further confirmed by the increase in TG content in MCAD-depleted HK2 cells (Figure 4B). Importantly, apoptosis in HK2 cells treated with PA and HG was no longer ameliorated by fenofibrate under the depletion of MCAD, based on the Bax/Bcl-2 ratio and the level of Cleaved caspase 3 (Figure 4C). TUNEL staining further confirmed the regulatory effect of MCAD on apoptosis in HK2 cells (Figure 4D and E). We concluded that MCAD contributes to the ameliorative effect of fenofibrate on lipid deposition and apoptosis in HK2 cells.

Fenofibrate Promotes MCAD Expression via the AMPK/FOXA2 Pathway

To investigate whether the effects of fenofibrate on MCAD expression and lipid accumulation are related to the AMPK/FOXA2 signaling pathway. We thus treated HK2 cells with AICAR (an AMPK activator), or compound C (an AMPK inhibitor). Similar to fenofibrate, AICAR significantly reduced lipid accumulation and TG contents compared to the PA- and HG- treated groups (Figure 5A and B). In contrast, the effect of fenofibrate in reducing PA- and HG-induced lipid accumulation was inhibited in the presence of compound C (Figure 5A and B). Both fenofibrate and AICAR increased phosphorylated AMPK, nuclear FOXA2 and MCAD expression in HK2 cells treated with PA and HG (Figure 5C and D). However, compound C reduced the protein levels of phosphorylated AMPK and MCAD and promoted the nuclear translocation of FOXA2 compared to the fenofibrate treated group (Figure 5C and D). In conclusion, these data suggested that fenofibrate regulates MCAD expression by activating the AMPK/FOXA2/MCAD signaling pathway to promote β -oxidation of fatty acids and ameliorate apoptosis.

Discussion

Increasing evidence has supported a more central role of renal tubules in DKD.²³ Compared to m/m mice, db/db mice showed severe histopathological damage and elevated apoptosis in the renal tubules. In this study, we demonstrated that lipid accumulation contributes to apoptosis in renal tubular cells, which is associated with decreased levels of MCAD expression in diabetic mice. Fenofibrate ameliorated DKD by activating the AMPK-FOXA2-MCAD signaling pathway and subsequently reducing renal tubular cell apoptosis.

Fenofibrate is currently used to treat hyperlipidemia and has been noted to improve diabetes, hypertension and I/R injury.²⁴ Past studies have demonstrated that fenofibrate improves insulin resistance, glucose control, and adiposity in db/db mice. Past studies suggested that fenofibrate treatment also reduced 24-hour urinary albumin excretion and improved renal histopathological changes, including reduced glomerular hypertrophy and thylakoid matrix expansion in db/db mice.^{25,26} However, a multinational, double-blind, randomized, controlled trial revealed that the incidence of adverse renal events was not lower among those who received pemafibrate than among those who received placebo among patients with type 2 diabetes, although pemafibrate lowered triglyceride, VLDL cholesterol, remnant cholesterol, and apolipoprotein C-III levels.²⁷ These results emphasize the importance of lowering atherogenic lipoprotein levels, rather than lowering triglyceride levels themselves. More research on the relationship between high triglyceride and type 2 diabetes still needs to be explored. In our study, fenofibrate improved renal function and alleviated renal pathological damage in db/db mice. Apoptosis plays a key role in renal tubular dysfunction.²⁸ Our work has demonstrated that

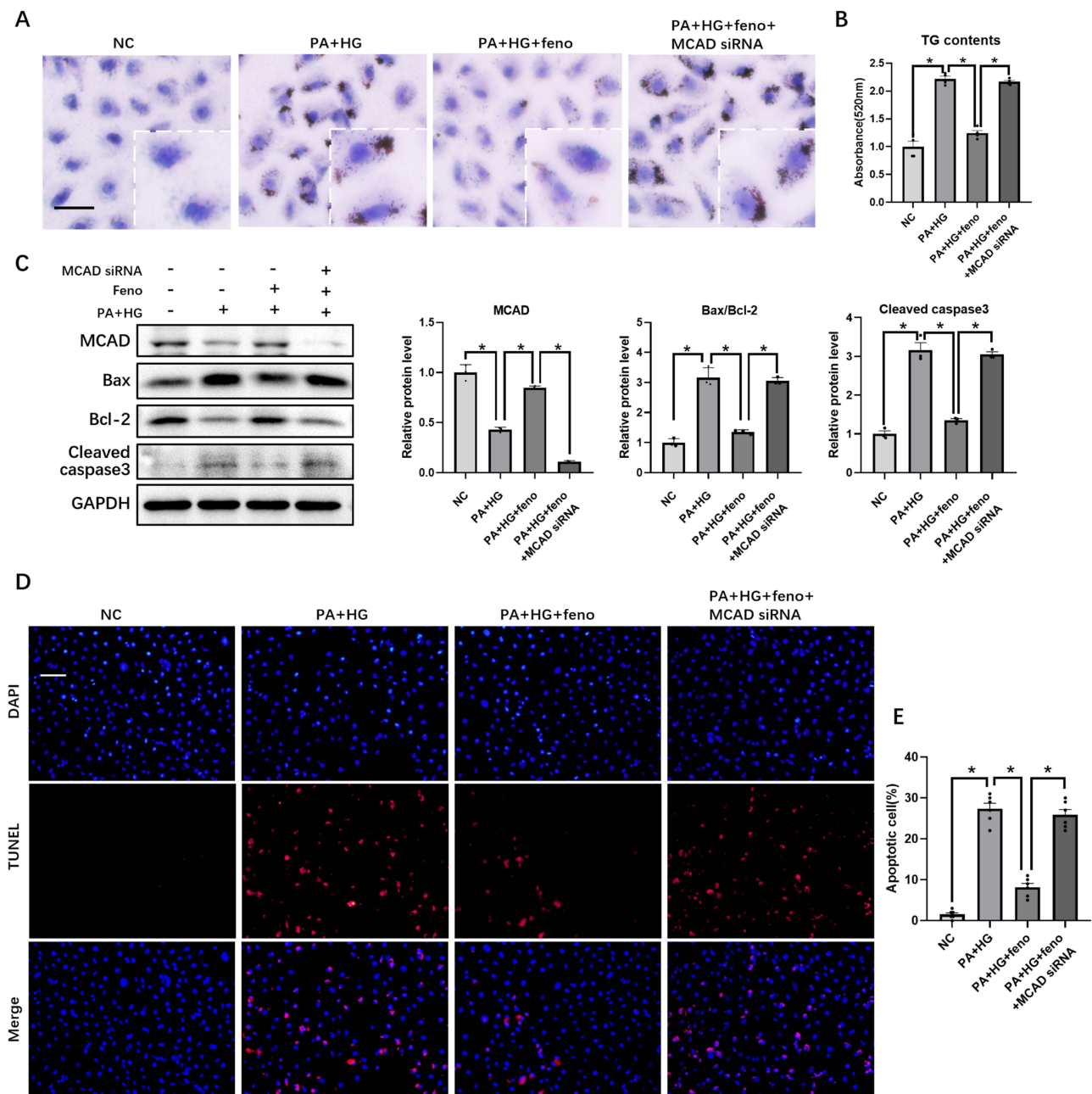


Figure 4 MCAD is required for fenofibrate to reduce lipid accumulation and apoptosis. **(A)** Representative images of Oil Red O staining and **(B)** Quantitative analysis of triglyceride (TG), $n=4$. Scale bar = 50 μm . **(C)** Representative blots and a quantitation graph of Bax, Bcl-2 and Cleaved caspase 3 protein expression in HK2 cells, $n=3$. **(D)** Representative images of TUNEL staining. Apoptotic cells are visualized as red and nuclei are stained with DAPI (blue), Scale bar = 100 μm . **(E)** Quantitative analysis of the number of TUNEL-positive cells. HK2 cells were transfected with or without MCAD siRNA and treated with or without 0.1 mmol/L PA and 25 mmol/L HG, with or without fenofibrate treatment. All data are presented as the means $\pm$ SEs. * $P < 0.05$.

Abbreviations: NC, negative control group; HG, high glucose; PA, palmitic acid; feno, fenofibrate; HK2 cells, human kidney proximal tubular epithelial cells.

apoptosis was exacerbated in both db/db mice and in PA- and HG-stimulated HK2 cells. Importantly, Western blot and TUNEL assays showed that fenofibrate attenuated apoptosis in the renal tubules of db/db mice. Apoptosis of renal tubular cells is probably attributed to the effects of lipotoxicity.²⁹ MCAD is involved in mitochondrial β -oxidation of fatty acids and its deficiency leads to lipid accumulation in multiple tissues and is potentially fatal, implying that MCAD may play a key role in lipid accumulation. A study has shown that MCAD depletion in primary GBM models induced an irreversible cascade of detrimental metabolic effects characterized by accumulation of unmetabolized MCFAs, which induced lipid peroxidation and oxidative stress, irreversible mitochondrial damage, and apoptosis.⁸ Interestingly, we

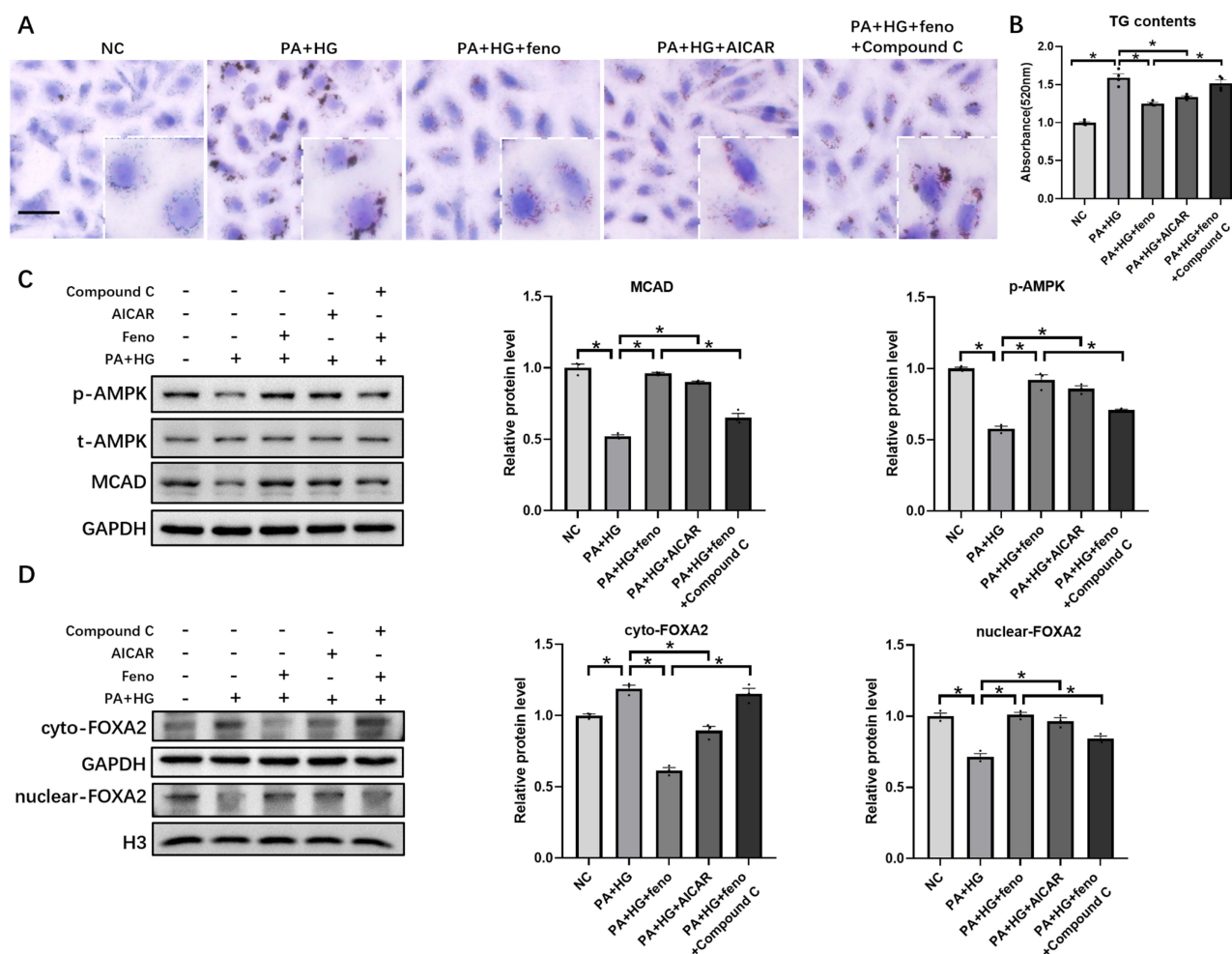


Figure 5 Fenofibrate promotes MCAD expression via the AMPK/FOX A2 pathway. **(A)** Representative images of Oil Red O staining and **(B)** Quantitative analysis of triglyceride (TG), $n=4$. Scale bar = 50 μm . **(C and D)** Representative blots and a quantitation graph of p-AMPK, t-AMPK, MCAD, cyto-FOX A2, nuclear-FOX A2 protein expression in HK2 cells, $n=3$. HK2 cells were treated with or without 0.1 mmol/L PA and 25 mmol/L HG, with or without fenofibrate, in the presence or absence of AICAR or compound C. All data are presented as the means $\pm$ SEs. * $P < 0.05$.

Abbreviations: NC, negative control group; HG, high glucose; PA, palmitic acid; feno, fenofibrate; cyto, cytoplasmic; HK2 cells, human kidney proximal tubular epithelial cells.

observed that inhibition of MCAD in HK2 cells induced lipotoxicity-induced apoptosis, suggesting that MCAD is a key factor in regulating renal tubular lipid metabolism and apoptosis. Fenofibrate treatment restored the expression of MCAD in db/db mice and a renal steatosis model and significantly reduced lipid accumulation and apoptosis in db/db mice. We provide a novel insight into the key role of MCAD in the regulation of fatty acid metabolism and apoptosis in the renal tubules. These results also provide a potential mechanism for the protective effect of fenofibrate against DKD.

We explored the potential mechanism by which fenofibrate impacts the activation of MCAD. AMPK is a serine/threonine protein kinase that regulates metabolic homeostasis, cell proliferation, and cell death. Activation of AMPK has been reported to improve DKD.^{30,31} Fenofibrate activates not only peroxisome proliferator-activated receptor- α (PPAR α) but also adenosine monophosphate-activated protein kinase (AMPK).²⁶ In vitro studies have demonstrated that fenofibrate can activate AMPK in retinal endothelial cells and myocytes, etc.^{32–34} Previous studies have found that activated AMPK inhibits FOXA2 phosphorylation and promotes FOXA2 nuclear translocation by inhibiting the PI3K/Akt pathway.³⁵ Wolfrum demonstrated that FOXA2 translocates to the nucleus and binds to the promoter of the MCAD gene, upregulating its transcription and reducing lipid accumulation in the liver.¹⁵ In our study, we found decreased nuclear FOXA2 and phosphorylated AMPK in db/db mice and in PA- and HG- induced HK2 cells, and those changes were reversed by fenofibrate treatment. Moreover, AICAR similar to fenofibrate, increased the expression of phosphorylated AMPK and MCAD and inhibited the cytoplasmic translocation of FOXA2. In contrast, compound C (AMPK

inhibitor) inhibited the effect of fenofibrate in increasing phosphorylated AMPK, MCAD and nuclear FOXA2 protein in PA- and HG-induced HK2 cells. These results demonstrated that the AMPK/FOXA2/MCAD pathway is critical for fenofibrate to ameliorate lipid accumulation and apoptosis in DKD.

Conclusion

In summary, our study demonstrated that fenofibrate upregulates the expression of MCAD to ameliorate β -oxidation of fatty acids and lipid accumulation through the AMPK/FOXA2 pathway. Furthermore, MCAD mediates the ameliorative effect of fenofibrate on renal tubular cell apoptosis. These data indicated that the AMPK/FOXA2/MCAD pathway plays a key role for fenofibrate to attenuate DKD both in vivo and in vitro, which provides a new insight supporting fenofibrate as an effective drug for DKD, and MCAD might be a therapeutic target of DKD.

Data Sharing Statement

All the data in the manuscript are available upon reasonable request from the corresponding author.

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Disclosure

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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