Original Research Article

Delayed treatment with fenofibrate protects against high-fat diet-induced kidney injury in mice: the possible role of AMPK-autophagy

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Running Headline: Effects of fenofibrate on AMPK and autophagy

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ABSTRACT

Fenofibrate activates not only peroxisome proliferator-activated receptor α (PPARα) but also adenosine monophosphate-activated protein kinase (AMPK). AMPK-mediated cellular responses protect kidney from high-fat diet (HFD)-induced injury, and autophagy resulting from AMPK activation has been regarded as a stress-response mechanism. Thus, the present study examined the role of AMPK and autophagy in the renotherapeutic effects of fenofibrate. C57BL/6J mice were divided into 3 groups: normal diet (ND), HFD, and HFD+fenofibrate (HFD+FF). Fenofibrate was administered 4 weeks after the initiation of the HFD when renal injury was initiated. Mouse proximal tubule cells (mProx24) were used to clarify the role of AMPK. Feeding mice with HFD for 12 weeks induced insulin resistance and kidney injury such as albuminuria, glomerulosclerosis, tubular injury, and inflammation, which were effectively inhibited by fenofibrate. In addition, fenofibrate treatment resulted in the activation of renal AMPK, upregulation of fatty acid oxidation (FAO) enzymes and antioxidants, and induction of autophagy in the HFD mice. In mProx24 cells, fenofibrate activated AMPK in a concentration-dependent manner, upregulated FAO enzymes and antioxidants, and induced autophagy, all of which were inhibited by treatment of compound C, an AMPK inhibitor. Fenofibrate-induced autophagy was also significantly blocked by AMPKα1 siRNA but not by PPARα siRNA. Collectively, these results demonstrate that delayed treatment with fenofibrate has a therapeutic effect on HFD-induced kidney injury, at least in part, through the activation of AMPK and induction of subsequent downstream effectors; autophagy, FAO enzymes, and antioxidants.

KEYWORDS: AMPK, Autophagy, Fenofibrate, High-fat diet, Kidney injury
INTRODUCTION

Obesity is an independent risk factor for the development and progression of chronic kidney disease (CKD) (16, 22, 36). Various factors, including hemodynamic, physiological, structural, and pathological changes, are involved in obesity-induced kidney injury (35). AMPK is a major sensor of cellular energy that serves to maintain metabolic homeostasis (11). In addition, the enzymatic activity of AMPK controls numerous enzymes and biomolecules that are associated with various cellular metabolic pathways. In the kidney, the downregulation of AMPK has been observed along with alteration in foot process effacement (15) and tubular injury (8). High-fat diet (HFD)-induced kidney diseases are alleviated by AMPK activation through regulation of the inflammatory response, oxidative stress, and lipid accumulation (7, 8). Downstream targets of AMPK include mammalian target of rapamycin (mTOR) and Unc-51-like kinase 1 (ULK1), which are the key autophagy initiation enzymes. Recently, autophagy has been regarded as an important therapeutic mechanism for various diseases, including kidney injuries (19, 33).

Fenofibrate is a fibric acid derivative that has been used worldwide for the treatment of dyslipidemia (42). Additionally, the protective effects of fenofibrate against obesity-induced CKD include decreases in renal fat accumulation, lipotoxicity, fibrosis, inflammation, and oxidative stress through PPARα signaling (1). However, the pharmacological effects of fenofibrate, such as the inhibition of inflammation, apoptosis, and lipid synthesis in HUVEC and retinal endothelial cells, are AMPK-dependent but PPARα-independent (17, 27). A recent study shows that AMPK plays an important role on fenofibrate-mediated improvement of kidney injury against lipotoxicity in db/db mice (13). Besides, AMPK is the key player in autophagy (9), and impaired autophagy has been suggested to play an important role in HFD-induced tubular injury in kidney (23, 44). ULK1, an autophagy initiation factor is regulated by mTORC1 and AMPK (18).
Considering all together, it may state that fenofibrate has pharmacologic effects through AMPK (2, 3, 13, 17, 27, 28, 30, 37, 41). Thus, the present study investigated the therapeutic mechanism of fenofibrate targeting link between AMPK and autophagy in obesity-induced kidney injury using mouse model. The data suggest that the therapeutic effects of delayed treatment with fenofibrate are mediated, at least in part, through AMPK activation which may lead to induction of autophagy, regulation of FAO, and upregulation of antioxidant enzymes.
MATERIALS AND METHODS

Materials. Chemicals, tissue culture plates, and immunoblotting antibodies were obtained from Sigma-Aldrich Company (St. Louis, MO, USA), Nunc (Rochester, NY, USA) and Cell Signaling Technology (Danvers, MA, USA), respectively, unless otherwise stated.

Animals. All animal experiments were approved by the Institutional Animal Care and Use Committee (2011-01-006) of Ewha Womans University. Eight-week-old male C57BL/6J mice were divided into 3 groups (7-9 mice/group): ND, HFD, and HFD+FF. Animals were fed either on ND (Harlan 2018S, Indianapolis, IN, USA) or HFD (Harlan TD06414) for 12 weeks. Fenofibrate was administered daily at a dose of 50 mg/kg body weight by oral gavage after 4 weeks on the HFD. Our preliminary data showed that there was no discernible toxic effect of fenofibrate at dose of 50 mg/kg body weight (data not shown). The ND and HFD mice were orally administered with an equal volume of 0.5% carboxymethyl cellulose (CMC) Mice were sacrificed after 12 weeks on the HFD. Before mice were sacrificed, urine samples were collected in a metabolic cage for 24 h to examine the urine protein excretion and creatinine levels. After anesthesia with 16.5% urethane (10 ml/kg), blood was collected in heparin-coated syringe. Mice were perfused through heart by diethylpyrocarbonate-phosphate buffered saline (DEPC-PBS, pH 7.4). The left kidneys were rinsed and weighed and then stored at -80°C for further analysis. The right kidneys were fixed via heart perfusion with freshly prepared 2% periodate-lysine-paraformaldehyde (PLP) fixative, pH 7.4 and kept at room temperature for subsequent analysis.

Measurements of blood parameters. The blood hemoglobin A1c (HbA1c) level was determined using the DCA2000 HbA1c reagent kit (SIEMENS Healthcare Diagnostics, Inc.,
Tarrytown, NY, USA). After centrifuging the blood sample at 900 g for 15 min at 4°C, the plasma was collected. The levels of plasma triglycerides, cholesterols, and free fatty acids were measured using an EnzyChrom™ colorimetric assay kit (BioAssay Systems, Hayward, CA, USA). The levels of plasma insulin were measured using enzyme-linked immunosorbent assay (ELISA) kits (Millipore Corporation, Billerica, MA, USA). Additionally, the levels of plasma adiponectin and IL-6 were measured using ELISA kits (R&D Systems, Minneapolis, MN, USA). Measurement of blood parameters were performed using assay kit according to respective manufacturer’s supplied protocols. Oral glucose tolerance test (OGTT) was performed at 4th and 12th week of HFD with or without fenofibrate treatment. Blood glucose was measured at each time point; 0, 15, 30, 60 and 90 min after oral administration of 2 mg/kg glucose by using glucometer (OneTouch Ultra, Johnson & Johnson co., CA, USA). In addition, plasma creatinine was measured using creatinine detection kit (Arbor Assays, Michigan 48108, USA).

Measurements of urine parameters. Urine samples were collected in a metabolic cage for 24 h before sacrifice and centrifuged at 3,000 rpm for 10 min. Urinary protein in the supernatants was analyzed by the Bradford method and SDS-PAGE (14), and all samples were assayed in duplicate to allow the mean value from a given mouse to be calculated. After electrophoresis, the 10% gel was stained with Coomassie Brilliant Blue (Bio-Rad Lab., Hercules, CA, USA) solution (0.2% Coomassie Brilliant Blue R-250, 50% methanol and 10% glacial acetic acid) overnight with gentle agitation. Further, the gel was destained in destaining solution (40% methanol and 10% glacial acetic acid) and the solution was replenished several times until background of the gel fully destained. Bovine serum albumin (BSA) was used as control. Images were taken by using HP4070 photosmart scanner. ImageJ software was used for densitometry analysis of the albumin protein band.
Urinary kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) were analyzed using mouse specific KIM-1 and NGAL ELISA assay kits purchased from R&D Systems, Inc. (Minneapolis, MN) (47) and Immunology Consultants Laboratory, (Portland, OR) (34), respectively. Urine aliquots were used in duplicate in each assay following the manufacturer’s recommended protocols.

Kidney morphometric analysis. 2% periodate-lysine-paraformaldehyde (PLP) fixed kidneys were removed, sliced transversely, and post-fixed overnight. After routine processing through graded alcohols and 100% xylene, the kidney tissues were embedded in paraffin and then sliced into 4 μm sections. The sections were stained with periodic acid-Schiff reagent to identify the basement membranes and glycogen deposition. From these sections, 30 different superficial glomeruli were randomly chosen for morphometric analysis. Images were photographed using Zeiss microscope-equipped Axio Cam HRC digital camera and Axio Cam software (Carl Zeiss, Thornwood, NY, USA). The total glomerular area, Bowman’s capsule area and FMA were determined using Image-Pro Plus4.5 software (Media Cybernetics, Silver-Spring, MD, USA) as described previously (14).

Further, deparafinized sections were stained with picrosirius red solution (0.5 g of sirius red F3B in 500 ml saturated aqueous picric acid solution) for 1 h to detect collagen deposition in kidney. The sections were rinsed quickly in 2 changes of acetic acid solution (5 ml acidic acid in 1 L of water). The sections were then rinsed in 100% ethanol for 5 sec and followed by dipping in xylene for 5 min. Finally, the clear sections were mounted in synthetic resin. Oil Red O staining was performed to evaluate lipid accumulation in kidney tissues as described (31). Images were photographed using Zeiss microscope-equipped Axio Cam HRC digital camera and Axio Cam software (Carl Zeiss). Images were quantified by using Image-Pro Plus4.5 software (Media Cybernetics) as described (14).
**Immunostaining.** Immunohistochemistry was performed using immunoperoxidase procedures and a commercially available kit (Dako, Glostrup, Denmark). The tissue sections were deparaffinized, and the endogenous peroxidase activity was quenched using a Dakoperoxidase solution for 30 min. The sections were then washed with PBS and incubated with a Dako serum-free blocking solution for 15 min. The sections were incubated with anti-collagen IV (1:200, Southern Biotechnology Associates, Inc., Birmingham, AL), anti-F4/80 (1:200, Santa-Cruz Biotechnology, Inc., Santa-Cruz, CA, USA), anti-p62 (1:400, MBL International, Woburn, MA 01801, USA), 8-oxo-dG (1:200, Trevigen, Gaithersburg, MD 20877 USA) and anti-nitrotyrosine (1:200, Santa-Cruz Biotechnology) overnight at 4°C. After washing in PBS, the sections were incubated with a LSAB2 kit (Dako) and then exposed to 3, 3′-diaminobenzidine (DAB) for 1 min. Images were photographed using Zeiss microscope-equipped Axio Cam HRC digital camera and Axio Cam software (Carl Zeiss). All images were quantified by using Image-Pro Plus 4.5 software (Media Cybernetics) as described (14).

**Cell culture.** Immortalized mProx24, mouse proximal tubule cells were supplied by Dr. Takeshi Sugaya (St Marianna University School of Medicine, Kanagawa, Japan). The mProx24 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% bovine serum (BCS; Gibco by Life Technologies, New Zealand), 100 U/ml penicillin, 100 μg/ml streptomycin, 20 mM glucose, and 44 mM NaHCO3 under a 5% CO2 environment at 37°C. The cultured cells (6×10^5 cells/well) grown in 6-well culture plate to confluence were growth arrested with DMEM containing 1% BCS for 24 h before commencing the experiments. Sub confluent mProx24 cells were treated with 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide (AICAR,
dissolved in distilled water), fenofibrate (dissolved in dimethyl sulfoxide, DMSO), compound C dissolved in DMSO, and palmitic acid bound to 10% BSA.

RNA interference. The mProx24 cells were grown at confluence of more than 50%. Cell growth was arrested with DMEM containing 1% BCS for 24 h and then, transfected with 35 nM siAMPKα1, (Bioneer, Daejeon, Korea) and siPPARα (ST Pharm Co., Seoul, Korea) using Lipofectamine RNAiMAX (Invitrogen) for 24 h. Scramble siRNA (Bioneer) was used as a negative control. The siRNA sequences were as follows: AMPKα1 sense: 5’-CCGACUUUUGUCUUUCAAA-3’ and antisense: 5’-UUUGAAAGACCAAAGUCGG-3’, PPARα sense: 5’-GUUCUUUHCAGCGAUAUUA-3’ and antisense: 5’-AUAGUUCGCCGAAAGCTT-3’. Then, the cells were pretreated with fenofibrate 30 min before the addition of 400 μM palmitic acid for 3 h.

Western blot analysis. The relative protein expression was analyzed using Western blot analysis as previously described (32). The tissue and cell lysates were separated using SDS-PAGE, transferred to a polyvinylidenedifluoride membrane, and probed with various antibodies, including anti-phospho-AMPKα (Thr172) (1:2000), anti-AMPKα (1:2000), anti-phospho-acetyl-CoA carboxylase (p-ACC) (Ser79, 1:2000), anti-ACC (1:2000), anti-AOX1 (1:1000, Santa-Cruz Biotechnology), anti-medium-chain acyl-coenzyme A dehydrogenase (MCAD, 1:1000, Santa-Cruz Biotechnology), anti-peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α, 1:2000, Santa-Cruz Biotechnology), anti-phospho-mTOR (Ser2448) (1:2000), anti-phospho-p70S6K (Thr389) (1:2000), anti-mTOR (1:2000), anti-phospho-ULK1 (Ser317) (1:1000), anti-phospho-ULK1 (Ser757) (1:2000), anti-ULK1 (1:1500), anti-LC-3B (1:2000), and anti-β-actin (1:5000, AbClon, Seoul, Korea) over night at 4°C on electric shaker. The blots were reacted with secondary antibodies for 1 h. After
washing, the membranes were incubated with enhanced chemiluminescence (ECL) detection reagent (Amersham Life Science) according to the manufacturer’s instructions. Positive immunoreactive bands were quantified for densitometry analysis using ImageJ software and normalized by β-actin.

Real time RT-PCR. Real time RT-PCR was performed to assess AMPKα1, acyl-CoA oxidase 1 (ACOX1), catalase, CPT1α, fibronectin, KIM-1, MCP-1, NGAL, plasminogen activator inhibitor-1 (PAI-1), PPARα, and Prx3. Isolation of total RNA from the whole kidney and mProx24 cells using TRIzol (Invitrogen) and the synthesis of cDNA were performed as previously described (32). Real time RT-PCR was performed using the SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA) and an ABI 7300 Real time RT-PCR Thermal Cycler (Applied Biosystems). The quantities of the test gene and internal control 18S mRNA were obtained from standard curves using Applied Biosystems software version 1.4.0. The mRNA expression levels of the test genes were normalized to the 18S mRNA levels. Primer sequences are shown in table 1.

Data analysis. All results were expressed as the mean±standard error (s.e.) with N as the number of experiments. The statistical significance of the differences among each group was compared by analysis of variance (ANOVA) and subsequent Fisher post-hoc analysis. The level for a statistical significance was set at a P<0.05.
RESULTS

Delayed treatment with fenofibrate ameliorates systemic insulin resistance and improves kidney function and morphology in HFD mice. To generate a diet-induced obesity model, 8-week-old C57BL/6J mice were placed on HFD for 12 weeks, and fenofibrate was administered 4 weeks after the initiation of the HFD to examine its therapeutic effect. Indices of renal injury such as macrophage infiltration, collage deposition, and oxidative stress marker proteins as well as Kim-1, Ngal, fibronectin (Fn1), and PAI-1(Pai1) mRNA were significantly increased at 4 weeks of HFD (Fig. 1A-E). We also confirmed that glucose intolerance was induced by the HFD for 4 weeks before the initiation of fenofibrate (Fig. 2A). 12-week HFD regimen caused an increase in body weight, which was accompanied by a 3-fold increase in the accumulation of peri-renal, subcutaneous, and epididymal fat in the HFD group compared to the ND group (Table 2). Administering fenofibrate effectively inhibited weight gain and the accumulation of these three types of fat. Glucose intolerance was further induced in the HFD group for 12 weeks and was effectively inhibited by a delayed treatment with fenofibrate (Fig. 2B). In addition, the HFD mice had higher plasma insulin, triglycerides (TG), and free fatty acids compared to those of the ND mice, and these plasma levels were inhibited by fenofibrate (Table 2). However, the plasma cholesterol was higher in both the HFD and HFD+FF mice than in the ND mice. Plasma adiponectin was increased in the HFD mice compared to the ND mice and was not affected by fenofibrate (Table 2). Plasma IL-6 was significantly increased in the HFD mice compared to the ND mice and decreased in the HFD+FF mice compared to the HFD mice (Table 2). Consistent with previous studies (24, 26), plasma creatinine level did not change among groups (Table 2). Proteinuria was measured by a Bradford assay and urine electrophoresis (Fig. 2C and D). Urinary protein excretion was significantly higher in the HFD mice compared to the ND
mice, whereas these levels were decreased in the HFD+FF mice compared to the HFD. These results suggest that HFD-induced proteinuria was improved by the fenofibrate treatment. The results of PAS staining showed that bowman’s capsule volume, the glomerular volume, and the fractional mesangial area were significantly increased in the HFD mice compared to that of ND, whereas these values were decreased in the HFD+FF mice compared to the HFD mice (Fig. 2E-H).

Picrosirius red and collagen IV immunohistochemical staining in kidney tissues were used to determine the regional change in collagen accumulation in the kidneys (Fig. 3A-D). The HFD mice exhibited increased collagen deposition in the tubulointerstitium and the glomerular mesangium compared to the ND mice, and this collagen deposition was effectively reduced by fenofibrate. Consistent with these results, mRNA expression of Fn1 and Pai1 in kidney was significantly increased in the HFD mice compared to the ND, and this expression was effectively inhibited by fenofibrate (Fig. 3I). The recruitment of F4/80 positive macrophages was significantly increased in the renal interstitial region of the HFD mice, and F4/80 positive cells were remarkably decreased in the HFD+FF mice (Fig. 3E and F). Consistent with this finding, the expression of MCP-1 (Ccl2) mRNA was also upregulated in HFD mouse kidneys, which was significantly diminished by fenofibrate (Fig. 3I). In addition, treatment of fenofibrate significantly reduced HFD-induced Kim-1 and Ngal mRNA upregulation in kidneys (Fig. 3J) as well as urinary excretion of KIM-1 and NGAL (Fig. 3K).

Since nitrotyrosine is an important marker of oxidative stress (5), we performed nitrotyrosine staining in the kidneys. Nitrotyrosine accumulation in the HFD mice was markedly increased both in the glomeruli and interstitium (Fig. 3G and H) compared to that of the ND mice, and fenofibrate effectively suppressed the renal nitrotyrosine accumulation observed in the HFD mice. These results demonstrate that HFD-induced kidney injuries, including fibrosis, macrophage infiltration, tubular injury, and oxidative stress, are inhibited by delayed...
treatment with fenofibrate.

Delayed treatment with fenofibrate activates renal AMPK. To examine whether fenofibrate activated AMPK in the kidney, phosphorylated protein levels of AMPK and its downstream target ACC were measured. Although being fed HFD for 12 weeks did not affect AMPK phosphorylation at Thr-172 under our experimental conditions (Fig. 4A), ACC phosphorylation was significantly reduced (Fig. 4B). Delayed treatment with fenofibrate increased the phosphorylation of AMPK at Thr-172 and ACC at Ser-79 in the HFD mice (Fig. 4A and B). Fenofibrate increased the phosphorylation of AMPK and ACC in a dose-dependent manner in mProx24 cells (Fig. 4C and D). A 30 μM dose of fenofibrate was shown to have a maximum effect on AMPK activation (Fig. 4C and D). Fenofibrate pretreatment also increased phosphorylation of AMPK and ACC in mProx24 cells treated with palmitic acid (Fig. 4E and F).

Delayed treatment with fenofibrate upregulates FAO enzymes and antioxidant enzymes through AMPK activation. We next evaluated the role of AMPK in the renotherapeutic effect of fenofibrate. The HFD did not alter renal ACOX1 (a key enzyme of peroxisomal FAO) but decreased MCAD (a key enzyme of mitochondrial FAO) and PGC1α (a key regulator of antioxidant enzymes) protein expression (Fig. 5A-C). Fenofibrate effectively increased the expression of each of these proteins (Fig. 5A-C). The mRNA expression of FAO enzymes, including ACOX1 and CPT1α (a rate limiting step of mitochondrial FAO), and antioxidant enzymes, including catalase (a predominant antioxidant enzyme located in peroxisome, scavenging H₂O₂) and Prx3 (an antioxidant enzyme located in mitochondria, scavenging H₂O₂), was not affected in the HFD mice compared to the ND, although fenofibrate treatment increased the mRNA expression of each of these factors (Fig. 5D). In addition, Oil Red O
staining revealed that lipid accumulation in the kidney was increased under HFD, which was effectively decreased by delayed treatment with fenofibrate (Fig. 5E).

To examine whether fenofibrate-induced FAO enzymes and antioxidant enzymes were mediated by AMPK activation, compound C at concentration of 20 or 40 μM was co-treated with 30 μM fenofibrate under palmitic acid stimulation. Fenofibrate upregulated the mRNA expression of ACOX1, CPT1α, catalase, and Prx3, which were effectively inhibited by compound C (Fig. 5F-I). These results imply that the effects of fenofibrate on the induction of FAO enzymes and antioxidant enzymes in cultured mProx cells were mediated by AMPK.

**Fenofibrate induces autophagy in an AMPK-dependent manner.** ULK1, an autophagy initiation factor, has been recently reported to be regulated by mTORC1 and AMPK (18). The HFD significantly increased the phosphorylation of mTOR at Ser-2448 (Fig. 6A). ULK1 phosphorylation at Ser-757, a downstream target of mTOR was increased in HFD kidney (Fig. 6B), although it did not reach to statistical significance. To confirm mTOR activity, we have checked phosphorylated p70S6K (Thr389), another target of mTOR. As expected, phosphorylation of p70S6K was increased under HFD, and fenofibrate treatment significantly inhibited HFD-induced phosphorylation of p70S6K (Fig. 6C). ULK1 phosphorylation at Ser-317, which is activated by AMPK, tended to be decreased under HFD, which was significantly increased after fenofibrate administration (Fig. 6D). The conversion of LC-3BI to LC-3BII, an indicator of autophagic activity (21), decreased in HFD and was significantly increased in the HFD+FF mice compared to the HFD (Fig. 6E). In addition, p62 immunoreactivity was higher in the kidney of HFD mice, which was effectively reduced by treatment with fenofibrate (Fig. 6F).

In mProx24 cells, fenofibrate also dose-dependently altered phosphorylation of mTOR and ULK1 at Ser-317 (Fig. 6G and H), which is consistent with the in vivo results. mTOR
phosphorylation was also suppressed by fenofibrate pretreatment in mProx24 cells cultured under palmitic acid stimulation (Fig. 6I). Treatment with chloroquine, a lysosomotropic agent that blocks autophagic flux, significantly increased LC-3BII accumulation, confirming that autophagy was induced by fenofibrate (Fig. 6J).

To further assess the role of fenofibrate-induced AMPK activation in the induction of autophagy, the effects of compound C and AMPKα1 siRNA on LC-3B conversion were examined. Under our experimental conditions, fenofibrate-induced LC-3B conversion was significantly reduced by compound C or AMPKα1 siRNA (Fig. 7A and D). The knockdown efficacy against AMPKα1 or PPARα was confirmed by their mRNA and protein levels (Fig. 7B, C and E). PPARα knockdown by PPARα siRNA did not change fenofibrate-induced LC-3B conversion, suggesting that the induction of autophagy by fenofibrate may not depend on PPARα (Fig. 7F).
DISCUSSION

The present study demonstrates that delayed treatment with fenofibrate effectively improves HFD-induced kidney injury along with activation of AMPK, upregulation of enzymes and regulators involved in FAO and antioxidant responses, and increases autophagy. Our *in vivo* results for HFD-induced insulin resistance and abdominal obesity, which are common indicators of the development of metabolic syndrome, were consistent with the results of many previous studies (29, 40). Body weight, peri-renal fat, epididymal fat, and subcutaneous fat were significantly increased by HFD, and treatment with fenofibrate effectively reduced these parameters to normal levels. Additionally, the HFD-induced insulin resistance and increased plasma insulin were ameliorated by delayed fenofibrate treatment. Significant kidney injuries, such as proteinuria, glomerular hypertrophy, mesangial expansion, glomerular fibrosis, macrophage infiltration, tubular injury, and oxidative stress, were induced by HFD as previously reported (29), and delayed treatment with fenofibrate effectively reversed these injuries, indicating the renotherapeutic effect of fenofibrate.

Evidence on the role of AMPK in renoprotection is increasing. A previous study showed that AMPK inactivation had been observed in the kidney after only 1 week on HFD, and treatment with AICAR, an AMPK activator, ameliorated HFD-induced urinary H$_2$O$_2$ and MCP-1 and renal MCP-1 upregulation, implying that reduced AMPK has an important role in mediating HFD-induced renal injury (7). In addition, the same group recently reported that a 14-week of HFD induced dysfunction of the lysosomal system and altered lipid metabolism, which was effectively normalized by AICAR treatment (8). On the other hand, several studies have reported that fenofibrate induces AMPK-mediated therapeutic effects in various tissues and cells (2, 3, 17, 27, 28, 30, 37, 41), and a recent study shows that fenofibrate improves diabetic kidney injury via AMPK activation (13). AMPK phosphorylation was not affected under our experimental condition, but AMPK activity as estimated by ACC and
ULK1 (ser317) phosphorylation was decreased in kidney at 12 weeks of HFD. The inconsistency between AMPK phosphorylation and its activity could be prompted by the characteristic of antibodies used or any other reasons which are not yet known. An important finding of the present study is that delayed treatment with fenofibrate upregulated the phosphorylation of AMPK (Thr172). Similar to the in vivo data, in mProx24 cells, fenofibrate upregulated AMPK and ACC phosphorylation in a concentration-dependent manner at basal or under palmitic acid stimulation.

Consistent with the previous study (39), fenofibrate significantly increased the protein or mRNA levels of ACOX, MCAD, and CPT1α which are involved with FAO, and antioxidant enzymes such as catalase and Prx3. We also confirmed that fenofibrate recovered the HFD-induced kidney lipid accumulation and downregulation of PGC1α, which is a co-activator protein known to interact with transcription factors that activate the expression of FAO and antioxidant enzymes (10). Further, fenofibrate induces PGC1α which results in increasing of antioxidant enzymes and downregulation of lipogenic enzymes in db/db mice (13). The FAO and antioxidant enzymes upregulated by fenofibrate are believed to be mediated by PPARα. However, we found that fenofibrate-induced ACOX1, CPT1α, catalase, and Prx3 mRNA expression in mProx24 cells were effectively inhibited by compound C in a dose-dependent manner, suggesting that lipid-lowering and anti-oxidant effects of fenofibrate are mediated in part by AMPK activation.

It is known that AMPK directly phosphorylates multiple sites on ULK1 (especially Ser317 and Ser777) and promotes the function of ULK1 in autophagy (3). However, mTORC1 directly phosphorylates ULK1 on Ser757, which is located in the AMPK binding motif on ULK1, and inhibits the association between ULK1 and AMPK. In HFD+FF mice, the phosphorylation of ULK1 at Ser317, a downstream target of AMPK was increased, and the HFD-induced phosphorylation of mTOR at Ser2448 was inhibited. ULK1 (ser757), a
downstream target of mTOR was not significantly different between HFD and ND groups. In order to confirm mTOR activity, we checked the phosphorylated p70S6K, another downstream target of mTOR. As expected, we found that phosphorylation of p70S6K increased under HFD condition and fenofibrate treatment significantly inhibited HFD-induced phosphorylation of p70S6K. These results suggest that AMPK-ULK1 pathway may regulate mTOR signaling. The inconsistency between mTOR phosphorylation and ULK1 ser757 phosphorylation could also be prompted by the characteristic of antibodies used or any other reasons which are not yet known. The AMPK-ULK1 pathway may result in an increased conversion of LC-3B in fenofibrate-treated HFD-fed mice kidneys. In addition, p62 accumulation, as indicator of autophagy disruption was also effectively decreased in fenofibrate-treated HFD-fed mice kidneys. Fenofibrate acted in a similar manner in mProx24 cells, leading to the induction of autophagy, which was further supported by an accumulation of LC-3BII when autophagic flux was blocked by chloroquine treatment.

To verify the role of AMPK in fenofibrate-induced autophagy, we further investigated the effect of the pharmacological or genetic suppression of AMPK. The fenofibrate-induced LC-3B conversion was effectively diminished by compound C or AMPKα1 siRNA. These observations suggest that AMPK may involve in the induction of autophagy. However, the inhibitory effect of AMPK suppression on fenofibrate-induced autophagy seems to be partial. These data suggest that other mechanisms by which fenofibrate-induced autophagy in tubular cell may exist. Further study is needed to investigate autophagy-related AMPK activation in response to fenofibrate at the molecular level in details. It should be noticed that we confirmed that PPARα siRNA had little effect on the LC-3B conversion ratio under our experimental condition, implying that fenofibrate-induced autophagy may be independent of PPARα activation.

Fenofibrate can inhibit protein kinase B (AKT) activation (4, 45) and consequently it may
reduce p-mTOR (ser2448) level. These reports are in agreement with the widely accepted concept of negative regulation of autophagy by phosphoinositide 3 kinase (PI3K)/AKT/mTOR signaling pathway (12, 43, 46). On the contrary, the growing evidences tend to show an AKT-independent pathway in autophagy activation (6, 20, 25). These conflicting results along with the notion that phosphorylated AKT was decreased in mice kidney under HFD model (38) suggest that the regulation of AKT is such a complex process. Thus, detailed mechanism involved in AKT activation and regulation of autophagy remained to be studied. Interestingly, our data are also in favor of AKT-independent pathway in autophagy activation. We found that HFD significantly reduced AKT activation and fenofibrate appeared to reduce HFD effect in mice kidney (data not shown). The present study had several limitations, which may have affected interpretation of the results. To dissect the role of PPARα or AMPK on fenofibrate-induced autophagy, using PPARα siRNA or antagonist, and PPARα or AMPK KO mice for in vivo experiments need to be investigated. Autophagy was assessed only by p62 staining, LC-3B protein conversion ratio, and lysosomal protease or fusion inhibitor (chloroquine) to monitor flux. Additional methods of assessing autophagy such as electron microscopic analysis are needed to validate our findings.

Overall, delayed treatment with fenofibrate has a therapeutic effect on HFD-induced kidney injury, at least in part, through the activation of AMPK and induction of subsequent downstream effectors; autophagy, FAO enzymes, and antioxidants. These novel mechanisms of fenofibrate may have important implications for the development of renotherapeutic strategies for obesity-associated kidney injury.

GRANTS

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DISCLOSURES

None of the authors declared any competing interest.

AUTHOR’S CONTRIBUTION

MS, KK, IH and HH were involved in conception and design of the experiments. MS, KK HK, HK, MJU, GL and JHL contributed to perform the experiments. MS, KK, MJU, HK, HK and JHL analyzed data. MJU, MS, KK and HH contributed to drafting the article or revising it critically for important intellectual content. HH made the final approval of the version to be published.
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Table 1. Primers used for real time RT-PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Product size (bp)</th>
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<td>ACOX1 (mouse)</td>
<td>Forward 5'-AGATTGGTAGAAATTGCTGC-3'</td>
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<tr>
<td></td>
<td>Reverse 5'-ACGCCACTTCCTTGCTCTTC-3'</td>
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<tr>
<td>AMPKα1 (mouse)</td>
<td>Forward 5'-AACGCATTTTGAGGACATGA-3'</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TTGTCCCGAAATCAGTGCAT-3'</td>
<td></td>
</tr>
<tr>
<td>Catalase (mouse)</td>
<td>Forward 5'-CACACCTACACGCGAGCAGGGCG-3'</td>
<td>156</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CTGCCGTCCCGAGTGAGGAGA-3'</td>
<td></td>
</tr>
<tr>
<td>CPT-1α (mouse)</td>
<td>Forward 5'-ACCACTGGCCGCATGCAAG-3'</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-AGCGAGTAGCAGCATGATCAT-3'</td>
<td></td>
</tr>
<tr>
<td>Fibronectin (mouse)</td>
<td>Forward 5'-TACCAAGGGTCATCCACACCCC-3'</td>
<td>366</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CAGATGGCAAAAAGAAGCAGAGG-3'</td>
<td></td>
</tr>
<tr>
<td>MCP-1 (mouse)</td>
<td>Forward 5'-CTTCTGGCCCTGCTGTCA-3'</td>
<td>127</td>
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<tr>
<td></td>
<td>Reverse 5'-CCAGCCTACTCATTTGGAATCA-3'</td>
<td></td>
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<tr>
<td>Ngal (mouse)</td>
<td>Forward 5'-GGCCAGTTCACTCTGAGAAA-3'</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TGTCGAACCTGTGTATCC-3'</td>
<td></td>
</tr>
<tr>
<td>Kim-1 (mouse)</td>
<td>Forward 5'-ACATATCGTCGAATCACAAGAC-3'</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CAACGAGGAGATGGGAGTGG-3'</td>
<td></td>
</tr>
<tr>
<td>PAI-1 (mouse)</td>
<td>Forward 5'-AGGGCTTCATGCCCCACTTCTCTCA-3'</td>
<td>192</td>
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<tr>
<td></td>
<td>Reverse 5'-AGTAGAGGGCCATTCCACAGCAC-3'</td>
<td></td>
</tr>
<tr>
<td>PPARα (mouse)</td>
<td>Forward 5'-CCTCAGGGGTACCTACGGA-3'</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GCCAATAGTTCCGGCGA-3'</td>
<td></td>
</tr>
<tr>
<td>Prx3 (mouse)</td>
<td>Forward 5'-GCGGCTCGGGAAGGTGTTC-3'</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TGCTGGGTGACAGCGGAGG-3'</td>
<td></td>
</tr>
<tr>
<td>18S (mouse)</td>
<td>Forward 5'-CGAAAGCATTTGCAAGAAT-3'</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-AGTCGGCCATCGTTATGGTC-3'</td>
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</table>
Table 2. Physiological characteristics at the end of the 12-week experimental period in the HFD mouse model with or without treatment of fenofibrate

<table>
<thead>
<tr>
<th></th>
<th>ND</th>
<th>HFD</th>
<th>HFD+FF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>29 ± 1</td>
<td>34 ± 1*</td>
<td>29 ± 1†</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>0.19 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>Peri-renal fat (g)</td>
<td>0.12 ± 0.05</td>
<td>0.69 ± 0.08*</td>
<td>0.37 ± 0.05*,†</td>
</tr>
<tr>
<td>Epididymal fat (g)</td>
<td>0.40 ± 0.06</td>
<td>1.33 ± 0.25*</td>
<td>0.75 ± 0.12†</td>
</tr>
<tr>
<td>Subcutaneous fat (g)</td>
<td>0.29 ± 0.04</td>
<td>0.97 ± 0.20*</td>
<td>0.53 ± 0.08†</td>
</tr>
<tr>
<td>Plasma triglycerides (μM)</td>
<td>226 ± 38</td>
<td>440 ± 49*</td>
<td>136 ± 59†</td>
</tr>
<tr>
<td>Plasma cholesterol (mg/dL)</td>
<td>55 ± 2</td>
<td>67 ± 0*</td>
<td>70 ± 1*</td>
</tr>
<tr>
<td>Plasma FFA (μM)</td>
<td>441 ± 34</td>
<td>686 ± 89*</td>
<td>395 ± 103†</td>
</tr>
<tr>
<td>Plasma adiponectin (μg/mL)</td>
<td>3.2 ± 0.1</td>
<td>4.0 ± 0.2*</td>
<td>4.6 ± 0.5*</td>
</tr>
<tr>
<td>Plasma IL-6 (ng/mL)</td>
<td>45.3 ± 12.2</td>
<td>112.3 ± 17.9*</td>
<td>57.2 ± 19.9†</td>
</tr>
<tr>
<td>Plasma creatinine (mg/dl)</td>
<td>0.83 ± 0.07</td>
<td>0.82 ± 0.03</td>
<td>0.95 ± 0.07</td>
</tr>
<tr>
<td>Hemoglobin A1c (%)</td>
<td>4.4 ± 0.1</td>
<td>4.6 ± 0.1</td>
<td>4.8 ± 0.1</td>
</tr>
<tr>
<td>Plasma insulin (μM)</td>
<td>0.3 ± 0.1</td>
<td>1.7 ± 0.6*</td>
<td>0.3 ± 0.1†</td>
</tr>
<tr>
<td>Calorie intake (kcal/day)</td>
<td>13.7 ± 0.3</td>
<td>13.3 ± 0.3</td>
<td>12.7 ± 0.2</td>
</tr>
</tbody>
</table>

ND, normal diet; HFD, high-fat diet; HFD+FF, HFD with fenofibrate. Data are the mean ± s.e.; n=7-9 in each group. *P<0.05, versus mice fed the ND and †P<0.05 versus mice fed with HFD.
**Figure legends**

Fig. 1. HFD induced kidney injury at 4 weeks of feeding. Representative photomicrographs of (A) picrosirius red, (B) collagen IV, (C) F4/80 and (D) 8-oxo-dG stained kidney sections. Original magnifications were x200 (picrosirius red, collagen IV; scale bar = 50 μm) and x100 (F4/80, 8-oxo-dG; scale bar=100 μm). Representative images are depicted. (E) mRNA expression of Kim-1, Ngal, Fn1, and Pai1 were determined by real time RT-PCR. ND, normal diet; HFD, high-fat diet. Data are the mean±s.e. of 7-9 mice in each group. *p<0.05.

Fig. 2. Fenofibrate prevented HFD-induced systemic insulin resistance, proteinuria, and glomerular injury. Oral glucose tolerance test (OGTT) was performed at 4th (A) and 12th week (B) of HDF treatment where fenofibrate was initiated after 4 weeks of HFD. Data are the mean±s.e. of 7-9 mice in each group. Proteinuria was measured by (C) Bradford assay and (D) urine electrophoresis. (E) Periodic acid-Schiff staining of kidney tissues indicates the (F) Bowman’s capsule and (G) glomerular volume, as well as the (H) mesangial expansion. Original magnification was x630. Scale bar=20 μm. Data are the mean±s.e. of 90 samples/group. ND, normal diet; HFD, high-fat diet; HFD+FF, HFD with fenofibrate. ND, normal diet; HFD, high-fat diet; HFD+FF, HFD with fenofibrate. *p<0.05.

Fig. 3. Fenofibrate prevented HFD-induced tubulointerstitial injury. Representative photomicrographs of (A) picrosirius red, (C) collagen IV, (E) F4/80, and (G) nitrotyrosine stained kidney sections. Original magnifications were x200 (picrosirius red, collagen IV; scale bar = 50 μm) and x100 (F4/80, nitrotyrosine; scale bar=100 μm). Quantitative analysis of (B) picrosirius red, (D) collagen IV, (F) F4/80, and (H) nitrotyrosine in 20 cortexes per mouse are depicted. mRNA expression of Fn1, Pai1 and Ccl2 (I), as well as Kim-1 and Ngal
(J) were determined by real time RT-PCR. (K) Urinary excretion of KIM-1 and NGAL proteins were measured by ELISA kit. Data are the mean±s.e. of 7-9 mice in each group.

*p<0.05.

Fig. 4. Fenofibrate phosphorylated AMPK and ACC. Fenofibrate administration upregulated (A) AMPK phosphorylation at Thr172 and (B) ACC phosphorylation at Ser-79 in kidney tissue. In mouse proximal tubule cells (mProx24 cells), a fenofibrate concentration-dependent test was executed with AICAR as a positive control to measure the phosphorylation of (C) AMPK and (D) ACC at 30 min. A pretreatment of 30 μM fenofibrate was applied 5 min before 400 μM palmitic acid stimulation for 25 min, and the protein levels of (E) AMPK and (F) ACC were measured. All protein levels were measured by immunoblotting. PA, palmitic acid; FF, fenofibrate. Data are the mean±s.e. of 7-9 mice in each group or of 4 experiments in the mProx24 cells. *p<0.05.

Fig. 5. Fenofibrate upregulated FAO enzymes and antioxidant enzymes through AMPK activation. Protein levels of (A) ACOX1, (B) MCAD and (C) PGC1α were measured in kidney tissues by immunoblotting. (D) mRNA levels of ACOX1(Acox1), CPT1α(Cpt1a), catalase, and Prx3(Prx3) were determined in kidney tissues were determined by real time RT-PCR. (E) Oil Red O staining was performed using kidney tissues, (Original magnifications were x200; scale bar = 50 μm). Further, mProx24 cells were co-treated with 30 μM fenofibrate and 20 or 40 μM compound C for 30 min, and the cells were then stimulated by 400 μM palmitic acid for 10 h. mRNA levels of (F) ACOX1, (G) CPT1α, (H) catalase and (I) Prx3 were determined by real time RT-PCR. PA, palmitic acid; FF, fenofibrate; CC, compound C. Data are the mean±s.e. of 7-9 mice in each group or 4 experiments in mProx24
Fig. 6. Fenofibrate induced autophagy. Phosphorylation of (A) mTOR, (B) ULK1 at Ser757, (C) p70S6K, (D) ULK1 at Ser317, and (E) the conversion of LC-3BI to LC-3BII were measured in kidney tissues. (F) p62 protein expression was determined by immunohistochemistry analysis in kidney tissues, (Original magnifications were x200; scale bar = 50 μm). In mProx24 cells, a fenofibrate concentration-dependent test was executed to measure the levels of phosphorylated (G) mTOR and (H) ULK1 at Ser317. (I) A pretreatment of 30 μM fenofibrate was applied 5 min before 400 μM palmitic acid stimulation for 25 min, and the phosphorylation level of mTOR was measured. (J) The mProx24 cells were pretreated with 30 μM fenofibrate and then treated with 400 μM palmitic acid and 10μM chloroquine for 3 h. All protein levels were determined by immunoblotting. PA, palmitic acid; FF, fenofibrate. Data are the mean±s.e. of 7-9 mice in each group or of 4 experiments in the mProx24 cells. *p<0.05.

Fig. 7. Fenofibrate induced autophagy in an AMPK-dependent and PPARα-independent manner. (A) The mProx24 cells were co-treated with 30 μM fenofibrate and 30 μM compound C for 30 min, and then the cells were stimulated by 400 μM palmitic acid for 12 h. (B-F) For AMPK or PPARα knockdown in mProx24 cells, siRNA oligonucleotides targeting the mouse AMPKα1 and PPARα mRNA were treated for 24 h. Then, the cells were pretreated with fenofibrate 30 min before the addition of 400 μM palmitic acid for 3 h. For AMPKα1 tests, mRNA levels of (B) AMPKα1 and PPARα, and (C) protein level of total AMPK were determined. Further, the LC-3BI to LC-3BII conversion ratio was calculated by immunoblotting assay (D). For PPARα siRNA test, (E) mRNA levels of PPARα and
AMPKα1 were measured. The LC-3BI to LC-3BII conversion ratio was calculated ($F$). The mRNA and protein expression were assessed by Real time RT-PCR and immunoblotting, respectively. PA, palmitic acid; FF, fenofibrate; CC, compound C; NS, no significance. Data are the mean±s.e. of 4 experiments. *$p<0.05$. 
Fig. 1

A. Picrosirius red staining showing differences between ND and HFD groups.

B. Collagen IV staining demonstrating variations between ND and HFD groups.

C. F4/80 staining highlighting differences in ND vs. HFD conditions.

D. 8-oxo-dG staining illustrating changes in ND and HFD conditions.

E. Bar graph showing gene expression of Kim-1, Ngal, Fn1, and Pai-1 in ND and HFD groups. Asterisks indicate significant differences.
Fig. 4

A

p-AMPK (Thr-172) t-AMPK

B

p-ACC (Ser-79) t-ACC

C

p-AMPK (Thr-172) t-AMPK

D

p-ACC (Ser-79) t-ACC

E

p-AMPK (Thr-172) t-AMPK

F

p-ACC (Ser-79) t-ACC
Fig. 7

A

LC-3BII
LC-3BII
β-actin

PA 400 μM - + + +
FF 30 μM - - + +
CC 20 μM - - - +

B

Relative increase (gene/18s)

Ampkα1 Ppara

Scramble + - + -
siAmpkα1 - + - -
PA 400 μM + + + +
FF 30 μM + + + +

C

t-AMPK

β-actin

Scramble - + + +
siAMPKα1 - - - -
PA 400 μM - + + +
FF 30 μM - - + +

D

LC-3BII
LC-3BII
β-actin

Scramble - + + +
iAmpkα1 - - - -
PA 400 μM - + + +
FF 30 μM - - + +

E

Relative increase (gene/18s)
Ppara Ampkα1

Scramble + - + -
siPPARα - + - -
PA 400 μM + + + +
FF 30 μM + + + +

F

LC-3BII
LC-3BII
β-actin

Scramble + - + -
iPPARα - + - +
PA 400 μM - + + +
FF 30 μM - - + +