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Acetyl-CoA carboxylase 2 suppression rescues human proximal tubular cells from palmitic acid induced lipotoxicity via autophagy

Wei Xin [a, 1], Xu Zhao [b, 1], Lei Liu [b], Ying Xu [c], Zhaoping Li [b], Liyong Chen [d], Xiaojie Wang [b], Fan Yi [e], Qiang Wan [c, *]

[a] Department of Central Laboratory, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, 250021, China
[b] School of Medicine, Shandong University, Jinan, 250012, China
[c] Department of Nephrology, Qianfoshan Hospital Affiliated to Shandong University, Jinan, 250014, China
[d] Department of Nutrition, Shandong Provincial Hospital Affiliated to Shandong University, Jinan, 250021, China
[e] Department of Pharmacology, Shandong University School of Medicine, Jinan, 250012, China

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ABSTRACT

Autophagy is a catabolic process that degrades damaged proteins and organelles in mammalian cells. Although acetyl-CoA carboxylase 2 (ACC2) plays a crucial role in the fatty acid metabolism, it keeps unknown whether ACC2 is associated with autophagic activity. The present work was designed to investigate the effects of ACC2 on palmitic acid (PA) induced lipotoxicity in human proximal tubular cells and the putative role of autophagy in this process. Here we show that autophagy was induced by PA in HK-2 cells. Moreover, the PA induced autophagy was regulated both by ACC2 suppression and CPTI inhibitor treatment, which represent an altered fatty acid β-oxidation. And the knockdown of ACC2 reduced PA-induced autophagy and thus protects the cells from PA-induced lipotoxicity with attenuated lipid accumulation and rescued cell viability. Collectively, the present study proposed a novel autophagy-involved mechanism of PA-induced renal lipotoxicity and provided potential therapeutic strategy by modulating lipid β-oxidation for diabetic nephropathy.

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1. Introduction

Lipids are essential constituents of all living cells serving as structural components and reservoir of energy, and their metabolism is precisely regulated. However, the ectopic accumulation of lipids may occur in non-adipose tissues when the lipids are overloaded and the balance of circular and cytosolic lipids is broken, which is termed as lipotoxicity [1], and is closely relevant to the pathogenesis of many metabolic syndromes [2], particularly to type 2 diabetes and its complications. Diabetic nephropathy (DN) is one of the major microvascular complications of diabetes mellitus and is the most common cause of end-stage renal diseases [3]. Accompanied by complex metabolic disorder, it is even more difficult to be cured than other kidney diseases.

Although numerous studies have described the lipid deposits in kidney, particularly in glomeruli and proximal tubes in a variety of both pathological and physiological conditions [4–7], whether the ectopic lipid accumulation is a cause or effect of renal injury in humans is still controversial. In 1982, Moorhead raised the hypothesis that chronic progressive kidney disease might be mediated by abnormalities of lipid metabolism, which offered new approaches to the study of kidney diseases by proposing a major pathogenetic role for lipid abnormalities [8]. This hypothesis shed a new light on the link between lipid accumulation and nephrotoxicity and led to further studies to investigate the roles of free fatty acids (FFAs) and lipid metabolism in kidney dysfunction. However, the underlying mechanisms of fatty acid metabolism in the pathogenesis of DN are not fully understood.

Fatty acid β-oxidation is a main pathway of FA catabolism and a major source for renal ATP production. Due to a high energy demand and relatively little glycolytic capacity, β-oxidation is particularly important for the proximal tubule cells [9,10]. Acetyl-CoA carboxylase (ACC) is a key enzyme in lipid metabolism and catalyzes the carboxylation of acetyl-CoA to malonyl-CoA [11].

* Corresponding author. 16766 Jingshi Road, Jinan, Shandong, 250014, China.
E-mail address: wanyanshaoqiang@163.com (Q. Wan).
[1] These authors contributed equally to this work.

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The ACC enzyme contains two isoforms, ACC1 and ACC2, that have distinct tissue distribution and cellular localization [11]. ACC1 is localized in the cytosol and ACC2 in the mitochondria. The ACC2 deficient mice have a higher FA oxidation rate, and less storage of fat [12], while the underlining mechanism has not been fully established.

Autophagy is a catabolic process that degrades damaged proteins and organelles in mammalian cells and plays a vital role in maintaining cellular homeostasis. The dysregulation of autophagy has been connected to many human diseases, including cancer, neurodegenerative, diabetic nephropathy and infectious diseases [13–15]. Autophagy is also an intracellular energy sensor and a cellular energy modulator. Although increasing evidence has shown that there is a link between autophagy and lipid metabolism [16,17], and autophagy may contribute to some metabolic syndromes, the potential role of autophagy in lipid-induced cellular dysfunction in renal tubule cells needs to be further clarified.

In this study, we demonstrated that autophagy was induced in human proximal tubular cells by palmitic acid (PA), which contributed to the PA induced cell death. Furthermore, we found that autophagy was regulated by FA β-oxidation and the suppression of ACC2 reduced PA-induced autophagy and thus protected human proximal tubule cells from PA-induced lipotoxicity with attenuated lipid accumulation and rescued cell viability. Collectively, the present study for the first time demonstrated that ACC2 mediate autophagic activity, which provides a novel molecular mechanism of PA-induced renal lipotoxicity. Pharmacological targeting of these signaling pathways may help design a new approach to develop therapeutic strategies for prevention of deterioration of kidney function in metabolic syndromes.

2. Materials and methods

2.1. Cell culture

The human proximal tubular epithelial cells (HK-2) were obtained from American Type Culture Collection (Rockville, MD), and cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified 5% CO_2 incubator at 37 °C.

2.2. Gene silencing of ACC2 by EGFP-ACC2-shRNA lentivirus transfection

For knocking down ACC2, specific four shRNAs targeting ACC2 and negative control shRNA with eGFP were designed and purchased from Genomeditech Company (Shanghai, China). The transfection was performed as previously described [18].

2.3. Atg5 siRNA transfection

The siRNA oligonucleotides targeting Atg5 and non-targeting control siRNA were obtained from Invitrogen and transfected into the cells with Lipofectamine 3000 according to the manufacturer's instructions.

2.4. Cell viability analysis and PI staining

The CCK-8 assay (Dojindo, Kumamoto, Japan) was used to determine cell viability as described. Propidium iodide (PI) staining (Nanjing KeyGen Biotech. Co. Ltd., Nanjing, China) was performed for 10 min at room temperature after washing cells gently with PBS. Analysis was done in fluorescence Microscope (Leica, Germany).

2.5. Western blotting

The procedure of western blotting was carried out as described previously [19]. The following primary antibodies were used: antibodies for SQSTM1/p62 (Cell Signaling, 5114), Atg5 (Novus Biologicals, NB110-53818), LC3B (Abcam, ab48394), Beclin-1 (Santa Cruz, SC-11427), ACC Sample kit (Cell Signaling, 12704S). Primary antibody against β-actin and horseradish peroxidase-conjugated secondary antibodies were from ZSGB-BIO.

2.6. Real time quantitative PCR (RT-qPCR)

Total RNA was isolated from HK-2 cells and reverse transcribed to cDNA by PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara). Real time PCR was performed using the light cycler 480 (Roche Diagnostics) with SYBR Premix Ex Taq™ II (Takara). The expression levels of the genes of interest were normalized to β-actin expression level. Primer sequences: Atg5 Fwd: TTTGCATCAC CTCTGCTTTC; Rev: TAGGCCAAAGGTTTCAGCTT. ACC2 Fwd: GATAGG CCATGTTTAGCACTGGTTG; Rev: CTCCAGCTGGGTACTTCC ATTCA.

2.7. Immunostaining studies

The immunofluorescence staining was performed as protocol modified from previous publication [20].

2.8. Oil Red O staining

Oil Red O was purchased from Sigma and the staining was performed as protocol modified from previous publication [21].

2.9. Electron microscopy

After treatments, the grouped HK2 cells were trypsinized and collected into centrifuge tubes after washing by PBS. Then, the cells were fixed by 3% glutaraldehyde at 4 °C, dehydrated by dimethylketone. After embedment in Epon-812, the samples were cut into ultrathin sections (70 nm). Uranium acetate and plumbum citrate dyed the ultrathin sections. They were observed with JEM-100sX electron microscopy.

2.10. Statistical analysis

Data are presented as means ± SD. T statistical analyses were performed with unpaired Student's t test. Three independent experiments were performed and $p < 0.05$ was considered to be significant.

3. Results

3.1. Palmitic acid induced lipotoxicity in HK-2 cells

HK-2 cells were treated with 300 μM PA (Sigma, coupled to BSA) for different time points, and BSA alone was used as a control. As shown in Fig. 1A, the lipid accumulation was observed as early as at 24 h after treatment and increased with the time duration, indicating a time-dependent intracellular accumulation of neutral lipid by Oil Red O staining. Consequently, the cell viability was detected by CCK-8 assay (Fig. 1B). There was no obvious difference between PA treated cells and BSA controls at 24 h after treatment. However, the cell viability was significantly decreased at 48 h, and was further reduced at 72 h, which was also confirmed by PI staining, that there were more PI positive dead cells after treated by PA for 72 h (Fig. 1C).

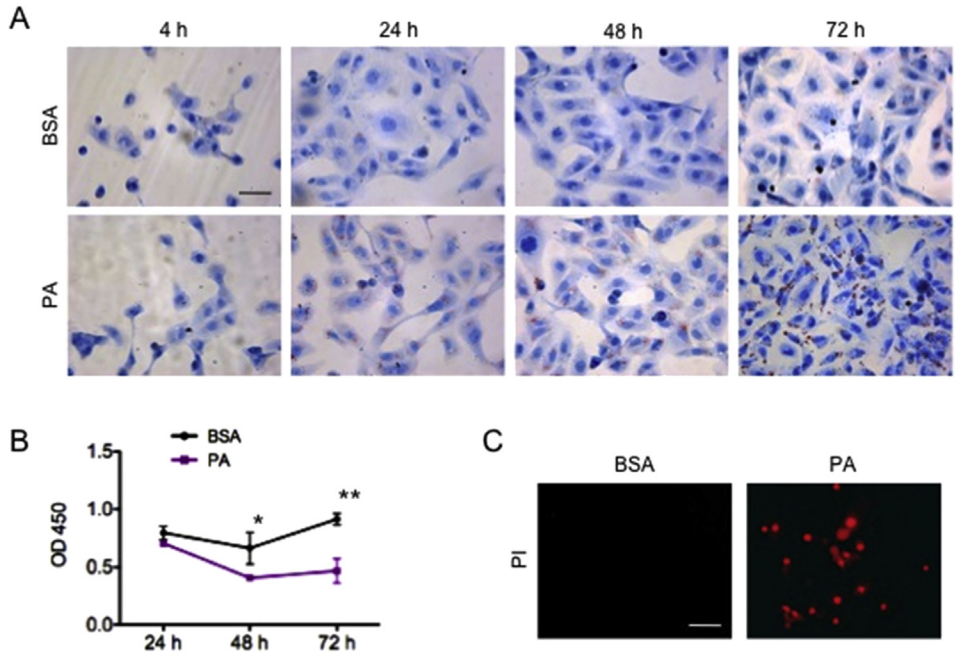

Fig. 1. Palmitic acid induced lipotoxicity in HK-2 cells. A) HK-2 cells were treated with 300 uM PA and the lipid accumulation was revealed by Oil Red O staining at indicated time of points. B) The cell viability was measured by CCK-8 assay. C) PI staining on HK-2 cells treated with BSA or 300 uM PA for 72 h. PA was coupled to BSA. Scale bar = 40 um. *P < 0.05, **P < 0.01.

3.2. PA induced autophagy in HK-2 cells which contributed to the reduced cell viability

First, the autophagy level was analyzed by western blotting as in Fig. 2A and we can see both LC3II/I ratio and Beclin-1 expression was increased, while p62 accumulation was decreased in a concentration dependent manner upon PA treatment. Second, the autophagic induction by PA was visualized by electron microscopy, which showed increased autophagosomes in PA treated cells (Fig. 2C). Third, we detected an additional increase of LC3II level after treating the cells with PA followed by chloroquine (CQ, Sigma) by both western blotting (Fig. 2D) and immunostaining (Fig. 2E), indicating an enhanced autophagic flux. Above all, these data clearly showed an autophagic induction and flux by PA treatment in HK-2 cells.

We further investigated the role of inhibiting autophagy in PA-induced lipotoxicity. Atg5 expression was suppressed by siRNA transfection and the knockdown efficiency was evaluated by both qPCR and western blotting (Suppl. Fig. 1). As shown in Fig. 2F, the cell viability was decreased by PA treatment, which was partially rescued by Atg5 knockdown approach as evidenced by CCK-8 assay.

3.3. Autophagy was regulated by FA oxidation

To clarify the contribution of FA β-oxidation in PA-induced autophagy, we treated the cells with either ACC2 shRNA lentivirus transfection to suppress ACC2 expression or with a CPTI inhibitor Etomoxir (Et, Sigma), which represents an enhanced or depressed FA β-oxidation respectively [18,22]. The ACC2 knockdown efficiency was evaluated by qPCR and western blotting (Suppl. Fig. 2). As shown in Fig. 3A, the immunostaining showed that PA treatment induced LC3 puncta formation, which was diminished by ACC2 knockdown and further increased by Et treatment.

This finding was also confirmed by western blotting that gene silencing of ACC2 inhibited PA induced autophagy as evidenced by decreased LC3 turnover, Beclin-1 expression and increased p62 accumulation. On the contrary, when the cells were treated with PA followed by Etomoxir, the autophagy level was elevated compared to PA treatment alone (Fig. 3B, C).

3.4. Suppression of ACC2 attenuated PA-induced lipotoxicity in HK-2 cells

Scramble shRNA transfected cells had obvious neutral lipid accumulation after being treated by PA for 72 h. However, it was dramatically reduced in ACC2 knockdown cells (Fig. 4A). The reduced cell viability by PA treatment was attenuated by ACC2 knocking down evidenced by CCK8 assay (Fig. 4B) and PI staining (Fig. 4C).

4. Discussion

Dysregulation of fatty acid (FA) oxidation has been implicated as a key nutrient-sensing pathway in the pathophysiology of many metabolic syndromes, especially obesity and diabetes mellitus. Diabetic patients show abnormal lipid metabolism [23] with increased plasma levels of free fatty acids. When renal lesions develop, the dyslipidemia will deteriorate the injury to the kidney [24]. However, the underlying mechanisms are not fully understood. Autophagy has captured extensive attention in recent years. Emerging evidence has indicated that autophagy regulates many critical aspects of normal and disease conditions. Notably, very recent studies have shown that autophagy contributes to lipid metabolism [25]. The autophagic alteration in different cell types under the metabolic stress induced by lipid accumulation has become a new hotspot. However, the effects of extra lipid accumulation on autophagy in renal cells have not been fully

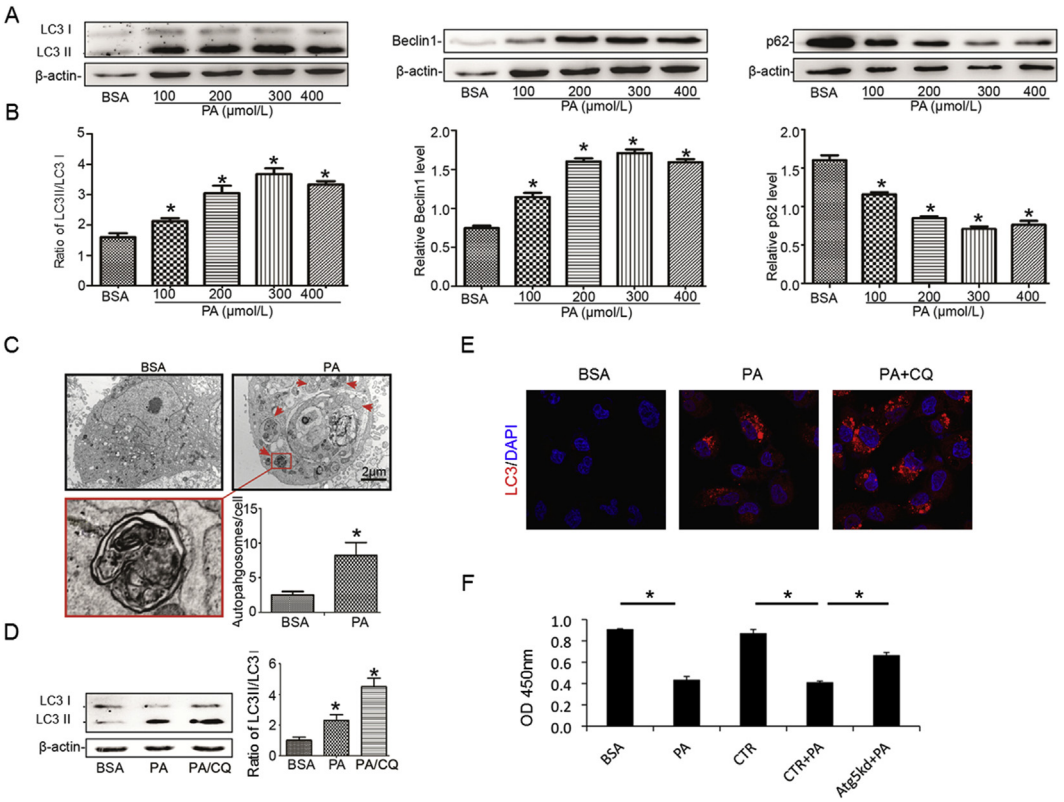

Fig. 2. Autophagy was activated in PA stimulated HK-2 cells. A) Western blotting for LC3, Beclin-1 and p62 in cell lysates treated with different amounts of PA for 2 h. BSA alone was used as control. B) Relative density of A. *P < 0.05 vs. BSA controls. C) Electronic microscopy pictures of HK-2 cells treated with BSA or 300 uM PA. Red arrows show autophagosomes. Bar = 2 um. D, E) Western blotting and immunostaining for LC3 in cells treated with 300 uM PA and 100 uM chloroquine (CQ). F) HK-2 cells were transfected with siRNA targeting Atg5 (Atg5 kd) or non-targeting control siRNA(CTR), and cell viability was measured by CCK8 assay after treated by 300 uM PA or BSA for 48 h *P < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

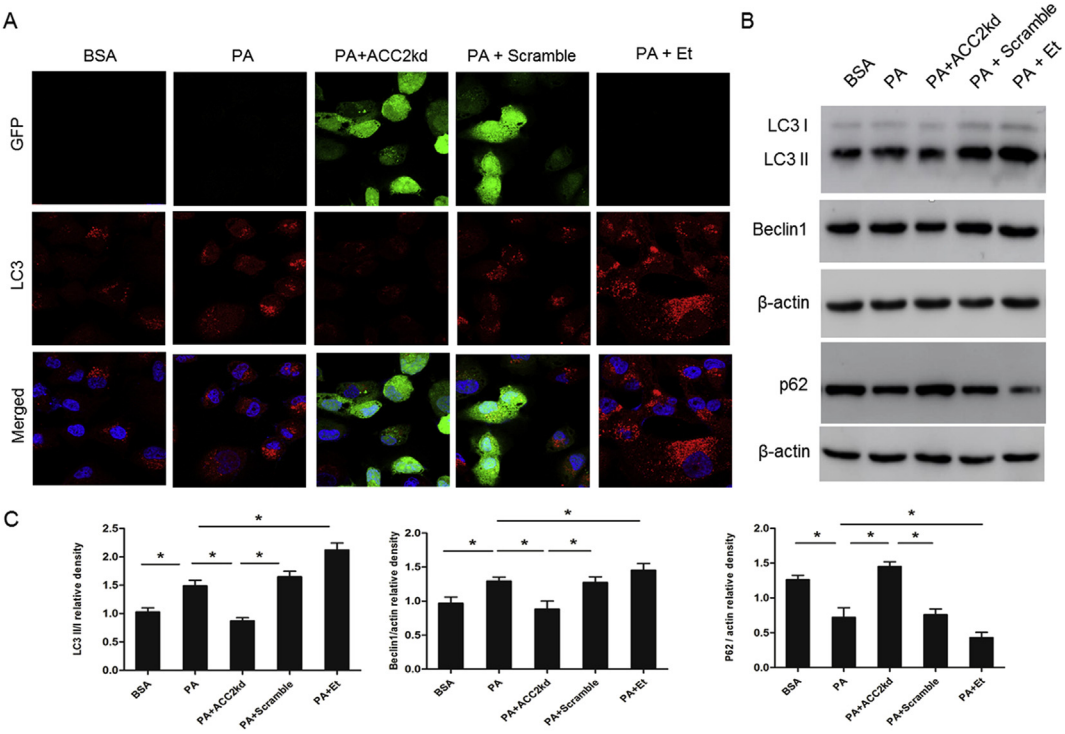

Fig. 3. Regulation of FA β-oxidation on autophagy. A) Immunostaining for LC3 in HK-2 cells transfected by LV-EGFP-shRNA targeting ACC2 or scramble shRNA treated by 300 uM PA and 80 uM Etomoxir. B) Western blotting for LC3, Beclin-1 and p62 in HK-2 cells treated by 300 uM PA and ACC2 shRNA knockdown or 80 uM Etomoxir. C) Relative density of B. *P < 0.05.

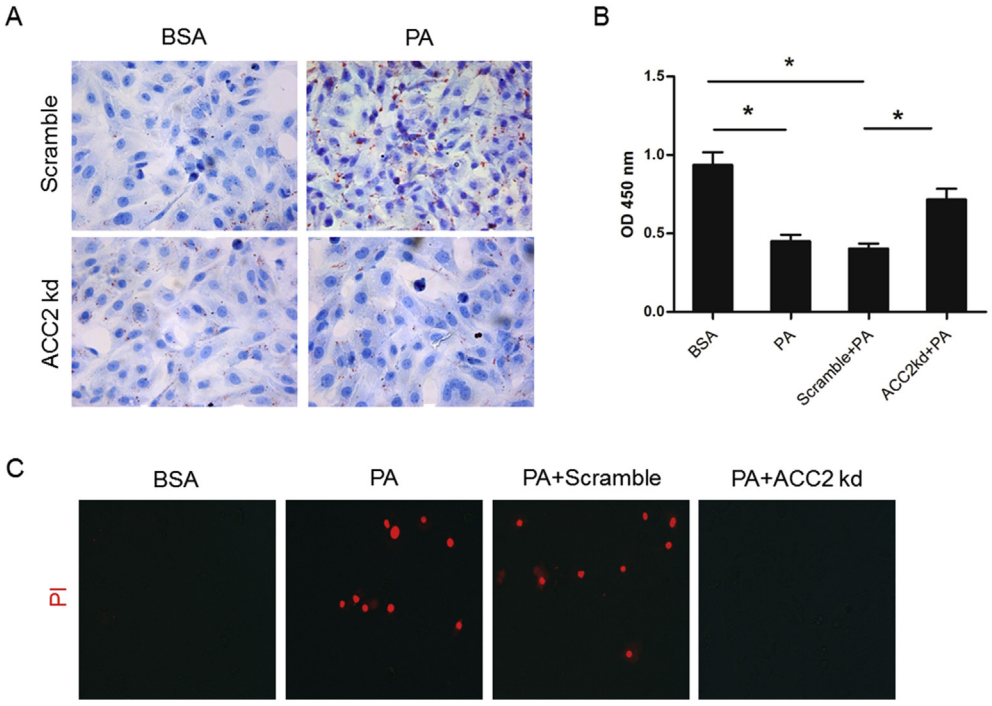

Fig. 4. ACC2 knockdown ameliorated PA induced lipotoxicity in HK-2 cells. HK-2 cells were transfected by scramble shRNA (Scramble) or specific shRNA targeting ACC2 (ACC2kd) and were treated by either BSA or 300 uM PA for 72 h. A) Oil red O staining was performed to investigate the lipid deposition in the cells B) Cell viability was measured by CCK8 assay. *P < 0.05. C) PI staining on HK-2 cells treated with BSA or 300 uM PA for 72 h after ACC2 was knocked down.

established, although the renal cells appear especially vulnerable to over-nutrition status [26].

One of the most important findings in this study is that PA induced autophagy in HK-2 cells, which contributes to the PA-induced cell death. In consistent with previous studies indicating that PA triggered autophagy in endothelial cells [19], mouse embryonic fibroblasts and hepatocytes [27], and autophagy was induced in the kidney from FFA-albumin overloaded mice [28], we found an enhanced autophagy level in PA-treated HK-2 cells evidenced by different methods, indicating an effect of disregulated FA β-oxidation on autophagy. However, there are controversial statements regarding to the function of autophagy in the PA-induced lipotoxicity process. In human hepatocellular carcinoma cells, autophagy was found to have a pro-survival role in PA-induced lipotoxicity and inhibition of autophagy sensitized the cells to PA-induced apoptosis [27]. However, Khan et al. showed that autophagy was the main trigger of PA-induced cell death of endothelial cells, and the inhibition of autophagy could rescue PA-induced cell death [19]. In the kidney, it has been shown that autophagy plays a protective role in kidney proximal tubules against acute kidney injury [29]. However, it remains unclear how autophagy is regulated and whether it contributes to chronic kidney injury induced by the lipotoxicity of PA overloading. Unexpectedly, we found that PA induced cell death of human proximal tubular cells via the initiation of autophagy in this study. In fact, a very recent study also reported that autophagy could be detrimental by inducing type II programmed cell death [30], especially when lysosomal dysfunction occurred [31].

More interestingly, we found that autophagy was regulated by FA β-oxidation. Autophagy is a highly conserved system in eukaryotic species for protein degradation through lysosomal machinery. It is classically regulated by nutrient related kinases such as mTOR, AMPK, PKC, PI3Ks [32,33]. In recent years, an increasing number of evidence has implied that autophagy may regulate lipid metabolism, especially lipolysis, and contributes to the physiological cellular responses to lipid over-accumulation [34]. Inhibition of autophagy in hepatocytes and MEF increased triglycerides levels and decreased FA oxidation rate; and in the liver specific Atg7 deficient mice, massive triglycerides were accumulated in the liver, indicating that autophagy participates in the regulation of hepatic lipid stores [15]. On the contrary, in adipocytes the loss of macroautophagy decreased the amount of lipid stored in adipose [17,35]. On the other hand, it was reported that changes in membrane lipid composition reduced autophagosome fusion [36], revealing that the alteration of lipid content had effects on autophagy formation. Based on the fact that lipid overloading can induce autophagy, FA β-oxidation may be able to regulate autophagy as a crucial nutrient-sensing pathway in lipid metabolism. However, there is no experimental evidence to the best of our knowledge. In this study, we used genetic knockdown targeting a key enzyme in FA β-oxidation, ACC2, to enhance FA β-oxidation and found that autophagy was reduced. Consistently, the treatment of Et, which can reduce FA β-oxidation further increased PA induced autophagy. These results clearly show that autophagy is precisely regulated by FA β-oxidation and the two processes are interplayed. It should be noted that although we did not explore the downstream signaling pathways involved in this process, acetyl CoA is considered as a potential candidate. Acetyl-CoA is an important metabolite of FA β-oxidation and a substrate of ACC2 [37], which can enter the citric acid cycle to supply energy, as well as a precursor for synthesis of many important compounds. Interestingly, taking up the central position in the metabolism, acetyl-CoA was found to be able to regulate the globle histone acetylation in yeast [38,39]. A very recent study from Schroede S et al. showed that nucleo-cytosolic acetyl-CoA linked the cellular metabolic state to the regulation of autophagy via effects on protein acetylation both in yeast and mammalian cells [40]. The more intensive mechanism needs to be explored in future work.

Collectively, we observed the PA induced lipotoxicity in renal tubular cells, and autophagy was induced by PA, which contributed to the PA-induced cell death. Furthermore, we demonstrated that autophagy was regulated by FA β-oxidation by the facts that upregulating FA β-oxidation by suppressing key enzyme ACC2 inhibited autophagy and downregulating FA β-oxidation by CPTI inhibitor stimulated autophagy, which represents a novel signaling pathway for autophagy regulation. Finally, The genetic knock down approach targeting ACC2 to increase FA β-oxidation could rescue the cells from PA-induced cell damage. Our findings may provide an innovative therapeutic strategy for treating patients with lipid overloading related diseases such as diabetic nephropathy and other metabolism syndromes.

Conflict of interest

None declared.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.05.070.

Transparency document

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