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Leonuride protects diabetic nephropathy by activating SREBPs pathway

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Abstract: Diabetic nephropathy (DN), a micro vascular complication of diabetes, is the main cause of end-stage renal disease, with a morbidity over 40% of diabetes. High glucose and lipid metabolism dysfunction are the leading cause of the development of DN. Previous study demonstrated that increased expression or activation of SREBPs in models of DN. Leonuride (LE), as an active constituent of *Leonurus japonicus* Houttuyn, has multiple biological activities, including antioxidant and anti-inflammatory effects. Previous studies showed that increasing the degradation of mature SREBPs is a robust way of lowering lipids and improve lipid metabolism dysfunction. However, effective regulation method of SREBPs degradation are still lacking. Herein, this study indicated that LE can effectively improve glucose and lipid metabolism disorders. In addition, the kidney function was also improved by inhibition of SREBPs activities in streptozocin (STZ)-induced type II diabetic mice. To our knowledge, this is the first time to describe the detailed mechanism of LE on the inhibition of precursor SREBPs, which would present a new direction for diabetic nephropathy treatment.

Keywords: TCM, SREBP, leonuride, diabetic nephropathy.

INTRODUCTION

Type 2 diabetes is a chronic disease caused by reduction of insulin-like effect and impaired insulin secretion. Insulin resistance and insulin deficiency could lead to glycolipid metabolism disorder in skeletal muscle, adipose tissue, liver, kidney and other tissues, which can cause various complications (Gloyn *et al*., 2018). Among them, DN is the most common disease, which also induces the global end-stage renal disease and mortality (Fang *et al*., 2021). At present, diabetic nephropathy treatment mainly depend on antihypertensive and hypoglycemic agents, including angiotensin converting enzyme inhibitor, insulin and oral hypoglycemic (Flyvbjerg *et al*., 2021). However, blood glucose control could merely delay the development of diabetic nephropathy, and the therapeutic effect is not satisfactory. Besides, renin-angiotensin-aldosterone inhibitors could raise the risk of hyperkalemia in DN patients. Therefore, it is urgent to develop safe and effective therapectic agents for the treatment of diabetic nephropathy.

Traditional Chinese medicine (TCM) exerts huge advantage in the treatment of complicated chronic diseases (Wu *et al*., 2021). TCMs owns the characteristic of "muti-components-multi-targets", which act on disease-related process in multiple ways. Therefore, there is urgent to find new agents for the treatment of diabetic nephropathy from TCM. Leonuride (LE) was demonstrated that it could greatly ameliorate hyperglycemia, hyperlipemia and renal injury of diabetic nephropathy (Yi *et al*., 2022; Lu *et al*., 2021). However, considering the complexity of diabetic nephropathy, we intend to clarify the anti-diabetic nephropathy efficacy of LE.

The pathogenesis of diabetic nephropathy has remained elusive (Wada *et al*., 2013). Recent studies have shown that it may be related to glycolipid metabolism disorder, hemodynamic changes, oxidative stress, inflammatory reaction, renal fibrosis. Among them, glycolipid metabolism disorder serves as the basis for the development of diabetic nephropathy, while oxidative stress is a key factor in progression to aggravate renal injury (Navarro-González *et al*., 2011; Papadopoulou-Marketou *et al*., 2017). As the absent of insulin-like effect on lipid synthesis and metabolism, fatty acid synthesis is enhanced while oxidation is inhibited, causing lipid accumulation in kidney (Su *et al*., 2020; Herman-Edelstein *et al*., 2014). However, the detailed mechanisms contributing to these effects remain unclear. Based on previous observations, we speculated that LE may exhibit anti-DN effects by regulating glucose and lipid metabolism dysfunction *via* inhibiting SREBPs activities (Zhang *et al*., 2021). Moreover, the potential mechanism of LE targeting to SREBPs to improve DN was focused.

In the present study, we revealed that LE effectively improves glucose and lipid metabolism disorders and kidney function by inhibition of SREBPs activities in DN mice. In addition, LE inhibits the transcriptional activity of SREBP was verified. To our knowledge, this is the first time to describe the detailed mechanism of SREBPs by LE.

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MATERIALS AND METHODS

Chemicals and reagents

Leonuride (98%) was purchased from Manster Biotechnology Co., Ltd. (Chengdu, China). Mouse micro albumin (mALB) ELISA assay kit, creatinine assay kit, urea assay kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Blood glucose meter and blood glucose teststrip were purchased from Johnson (USA). Citric acid and sodium citrate were purchased from Ron (Shanghai, China). Streptozotocin (STZ) was purchased from Sigma-Aldrich (USA). Firefly luciferase reporter gene detection kit and lipo6000 transfection reagent (Beyotime Biotechnology, Shanghai, China), SREBPs luciferase reporter gene plasmid (Jiman Biotechnology Co., Ltd., Shanghai, China) were used to construct luciferase reporter gene system. SREBP-1 antibody (Santa Cruz, USA), β-actin antibody (Santa Cruz, USA) were used for western blot experiment.

Cell culture

Human kidney 2 (HK-2), a proximal tubular cell line was obtained from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China).

Oil red O staining

Add 0.25g of oil red O powder to 50 mL of isopropanol to prepare a stock solution. Cells in 6-well plate were washed with PBS buffer and fixed with 1.5mL 4% paraformaldehyde for 30 mins. Dilute stock solution with water in a ratio of 3:2 (v/v) and filter with filter paper for twice. The fixed cells were then stained with 1.5mL of oil red O dilution for 20mins at room temperature, rinse with 75% ethanol for twice before imaging. Lipid droplets intensities of each image were counted with Image.

Nile red staining

Cells were washed with PBS buffer and then incubated with a final concentration of 100ng/ml nile red at 37℃ for 10min. Before imaging, cells were washed with PBS buffer again and immediately imaged under fluorescence microscope for analysis.

Measurement of luciferase reporter gene activity of SREBPs

Cells transfected with SREBPs and Renilla luciferase reporter plasmids for 24h and then assayed using dual luciferase assay kit (Vazyme Biotech, China) according to the protocol.

Animals and treatment

7 weeks old male C57BL/6J mice were purchased from SPF Biotechnology Co., Ltd., Beijing, China. All mice studies were approved by the Animal Ethics Committee of Taian City Central Hospital (No. TH202005). After adaptive feeding for one week, mice were feed with 60% high-fat diet (HFD, TP23400) and 10% low-fat control diet (TP23402) (Nantong Trophy Biotechnology Co., Ltd., Nantong, China) for 4 weeks and injected with either 0.1M sodium citrate buffer or streptozotocin (STZ) that dissolved in sodium citrate buffer once at a low-dose of 100mg/kg. After one week of injection, diabetic mice with fasting blood glucose ≥250mg/dl (13.8mM) were included in this experiment.

Mice were divided into 3 groups: chow group, HFD/STZ group and LE treatment group (60mg/kg/d).

Mice were placed in metabolic cages to collect 24h urine for biochemical analysis 1 week before sacrifice. Fasting blood glucose was measured from tail tips at the last week. After administration of 10 weeks, plasma was collected for biochemical analysis. The right kidneyswas fixed in 4% paraformaldehyde for pathological analysis, and the left ones were weighed for kidney index and then stored at -80°C for further study.

Pathological analysis

Mice kidneys were evaluated with hematoxylin-eosin (H&E) staining. Kidneys were also analyzed by periodic acid-schiff (PAS) staining.

STATISTICAL ANALYSIS

Graph pad prism 6 was generated to analyze all parameters with unpaired t-test. Results of animal and cell experiments were expressed as Mean ± SD, respectively. $P<0.05$ considered as significant.

RESULTS

LE improves glucose and lipid metabolism disorders in DN mice

STZ-induced type II diabetic mice model was used to appraise the effect of LE on glucose and lipid metabolism dysfunction (Erbatur *et al*., 2017). After 4 weeks of HFD feeding to induce insulin resistance, 100mg/kg STZ was injected intraperitoneally. To determine the hypoglycemic effect of LE on DN mice, fasting blood glucose and insulin were examined after 11 weeks of administration of LE. As expected, LE decreased fasting blood glucose significantly compared with the model group (fig. 1A-B). Taken together, it indicated that LE improves glucose level in DN mice. Furthermore, kidney H&E pathological sections showed that lipid steatosis remarkably increased in DN mice, and LE reversed lipid accumulation of kidney (fig. 1C). These results confirmed that LE effectively improves glucose and lipid metabolism disorders in DN mice.

LE improves kidney function and renal lipid accumulation in DN mice

Next, the kidney function in the progression of DN mice was assessed. In model group, the ratio of kidney weight

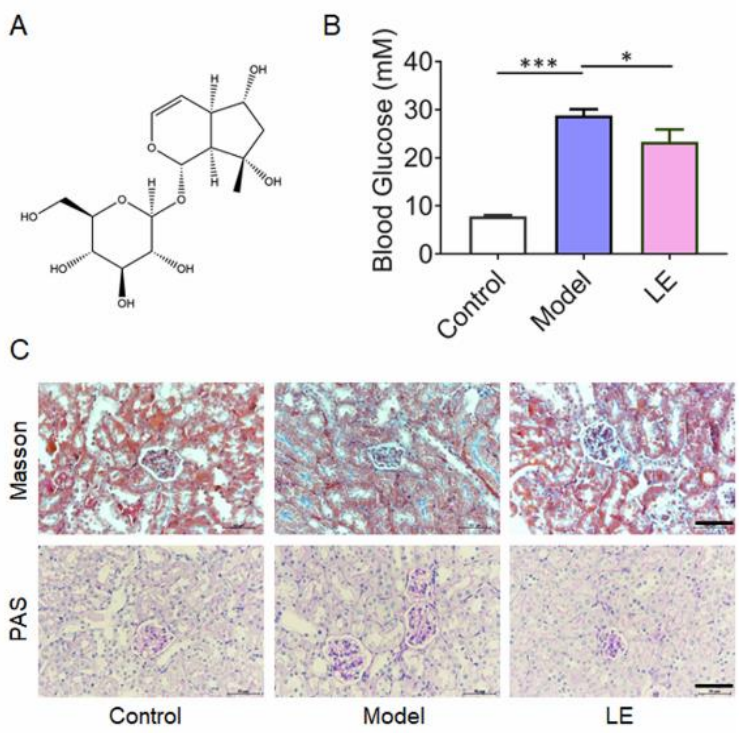

Fig. 1: Leonuride protects the Kidney *in vivo*. (A) The structure of Leonuride. (B) Fasting blood glucose was measured (n=6). (C) Kidney tissue sections were subjected to PAS and Masson's trichrome. A representative image from each group is shown (n=6). scale bar 100μm. *p≤0.05, ***p < 0.001.

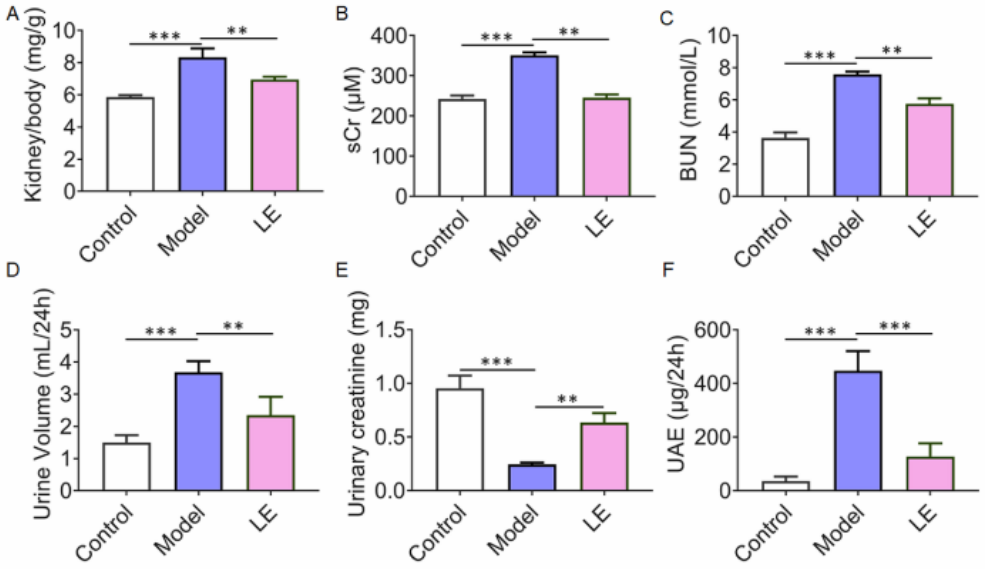

Fig. 2: Renal protective effect of LE on type 2 diabetic nephropathy. (A) The ratio of Kidney weight and body weight. (B) Levels of sCr. (C) Levels of BUN. (D) Levels of Urine volume. (E) Levels of Urinary creatinine. (F) Levels of UAE. (n=6). Error bars represent SD. **p<0.01, ***p<0.001.

and body weight was increased, and LE treatment decreased the ratio significantly (fig. 2A). In the following study, the index of renal function, such as creatinine (Cr) and blood urea nitrogen (BUN) were examined. Compared with the chow group, sCr and BUN significantly increased, and LE decreased the level (fig 2B-C). The volume of urine was increased in the model group and reduced by LE significantly (fig. 2D). Creatinine in urine was also decreased after LE administration (fig. 2E), Meanwhile, in the model group, urinary albumin excretion (UAE) was increased (fig. 2F).

LE ameliorated lipid accumulation in vitro

Furthermore, the efficacy on lipid accumulation of LE was also evaluted *in vitro*. Nile red and oil red O staining were performed to visualize the accumulation of lipid droplets when cells stimulated with an unsaturated fatty acid oleic acid (OA). As shown in fig. 3 and 4, lipid droplets were significantly decreased under cells administrated with fatty acid. Besides, the increased lipid droplets were almostly reversed to normal level when cells treated with LE. Taken together, LE improved kidney function in STZ-induced diabetic mice, which is type II diabetic nephropathy.

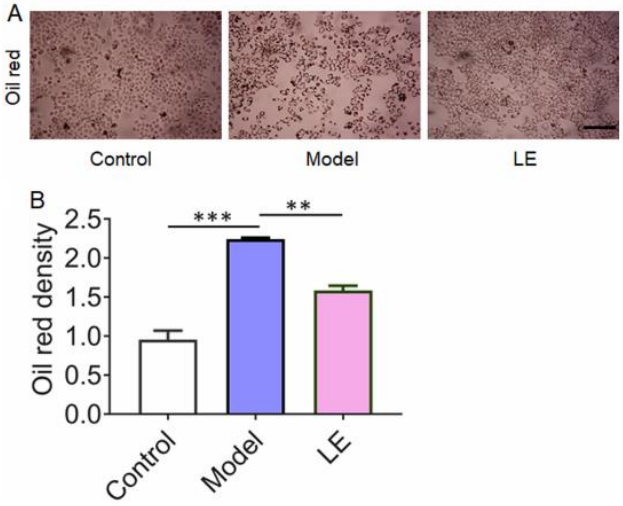

Fig. 3: LE ameliorated lipid accumulation in vitro. OA-induced HK-2 cells were treated with LE (10μM) or vehicle. (A) Oil Red O staining of HK-2 cells. scale bar 100 μm. (B) Quantification of 10 Oil Red O. (n=6). **p < 0.01, ***p < 0.001.

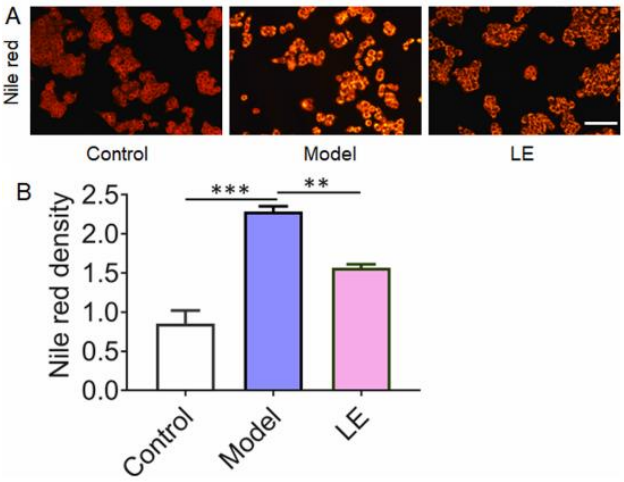

Fig. 4: LE ameliorated lipid accumulation in vitro. PA-OA-induced HK-2 cells were treated with LE (10μM) or vehicle. (A) Nile Red staining of HK-2 cells. scale bar 100μm. (B) Quantification of Nile Red. (n=6). **p < 0.01, ***p < 0.001.

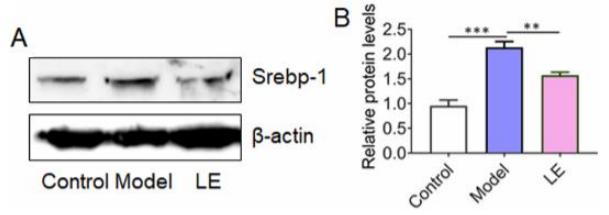

Fig. 5: (A) Western blot analysis and quantification of mature SREBP-1.

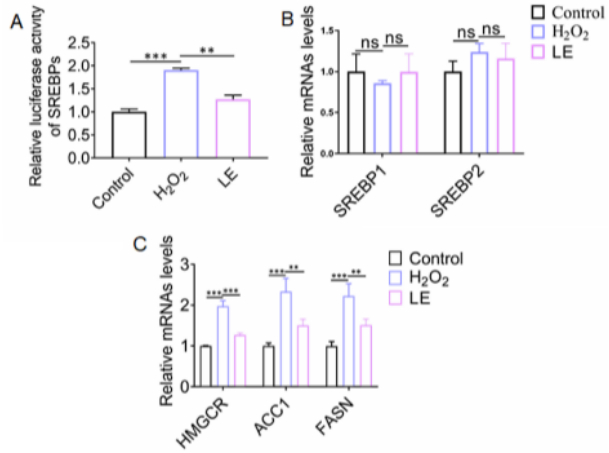

Fig. 6: (A) The relative SREBPs-Luc reporter activity. (B) The mRNA levels of SREBP1 and SREBP2 in kidney tissue. (C) The mRNA levels of SREBP target genes in kidney tissue.

LE inhibits SREBPs activities and lipid biosynthesis in vivo

SREBPs is a key transcription factor to regulate lipid homoeostasis (Xu *et al*., 2020; Jeon *et al*., 2012). To further explore the underlying mechanism of LE on the improvement of lipid accumulation and DN in DN mice, we investigated the expression of SREBPs and target genes involved in fatty acids and cholesterol synthesis in the liver and kidney tissues. The protein levels of SREBPs were elevated in the model group and LE significantly decreased SREBPs expression in a dose-dependent manner (fig. 5).

LE attenuated the transcriptional activity of SREBPs

Moreover, the SREBPs luciferase reporter gene system was constructed to assess the activity of SREBPs *in vitro* (Zhu *et al*. 2019). Besides, SREBPs activity increased significantly after OA stimulation in the SREBPs luciferase reporter gene system and decreased by LE (fig. 6A). Meanwhile, the gene expression of SREBP-1 and SREBP-2, which regulates cholesterol synthesis, were recorded. However, no significant difference was found (fig. 6B). In addition, target genes of SREBPs, which regulates synthesis of fatty acids, were significantly reduced with LE treatment (fig. 6C). Thus, it indicated that LE may improve glucose and lipid metabolism disorders and kidney function by inhibiting SREBPs and lipid biosynthesis *in vivo*.

DISCUSSION

Herein, our study identified LE as a bioactive component to treat DN. In addition, this study provides significant insights into the treatment of DN, a critical microvascular complication of diabetes. As the main cause of end-stage renal disease, DN's impact on the diabetic population, with a morbidity rate exceeding 40%, underscores the urgent need for effective therapeutic interventions. The central role of high glucose and lipid metabolism dysfunction in the development of DN has been a focal point of research, and the current study contributes notably to this body of knowledge. Previous research has identified the upregulation and activation of SREBPs in DN models, highlighting their potential as therapeutic targets. The present study's emphasis on LE, derived from Leonurus japonicus Houttuyn, is particularly noteworthy. LE's antioxidative and anti-inflammatory properties have been well-documented, and its role in enhancing the degradation of mature SREBPs offers a promising avenue for managing lipid metabolism dysfunction in DN. LE improves lipid metabolism disorder *via* down regulating SREBP-1 pathway. LE exerts prominent therapeutic potential for kidney dysfunction, which is of great significance to protect diabetes from progressing into end-stage renal disease. Measurements of albuminuria and serum creatinine are clinically performed to detect and stage kidney disease (Al-Oanzi *et al*., 2023; Fang *et al*., 2023). In the HFD/STZ-induced DN model, the levels of albuminuria and serum creatinine were markedly increased. LE administration significantly reversed the increased albuminuria and serum creatinine, which exhibits a protective effect against kidney dysfunction in DN. Notably, the beneficial effects of LE on kidney may suggest an effective strategy to chronic renal disease. Accumulated evidence confirmed that LE could regulate lipid metabolism through inhibiting lipid synthesis, activating fatty acid β-oxidation and repressing lipolysis in adipose tissue (Zhang *et al*., 2021). Consistently, our data demonstrated that LE alleviated lipid accumulation both in OA-induced high fat model and in STZ-induced diabetes mellitus. Prolonged high blood glucose induced mitochondrial dysfunction and promoted overproduction of ROS during the progression of DN. In addition, increased ROS has been reported to alter cholesterol metabolism and aggravate hepatic lipid accumulation and metabolic abnormalities during aging (Seo *et al*., 2019). In this context, simply reducing lipid content is not sufficient to recover lipid disorder and it seems to be equally necessary to clear away ROS. SREBP-1 is an important regulator accounting for lipid metabolism (Xu *et al*., 2022; Dorotea *et al*., 2020). In the present study, LE exerted a greater effect in reducing mature SREBP levels and target gene levels *in vitro*. But the mechanism how LE reduce the levels of SREBP-1 needs further investigations. In conclusion, this study not only enhances our understanding of the pathophysiology of DN but also opens up new therapeutic possibilities with LE's role in regulating SREBPs. It sets the stage for further research and clinical trials, which could lead to more effective and targeted treatments for patients suffering from this debilitating complication of diabetes.

CONCLUSION

In summary, this research attended to screen a synergy component for LE to promote the degradation of SREBP-1 in the treatment of type 2 diabetic nephropathy. We firstly focused on the association between oxidative stress and lipid synthesis mediated by SREBP-1 and recognized that LE could decrease the stability of mature SREBP-1. Furthermore, LE could ameliorate glucolipid metabolism, renal function and histopathological changes in type 2 diabetic nephropathy mice.

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