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Lithocholic Acid Ameliorates Diabetic Nephropathy by Suppressing the AGEs/RAGE Signaling Pathway and Modulating Inflammation, Endoplasmic Reticulum Stress, and Lipid Metabolism

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ABSTRACT: Diabetic nephropathy (DN) is a leading cause of end-stage renal disease, and the advanced glycation end products (AGEs) and their receptor RAGE play a critical pathogenic role in DN progression. This study investigated the therapeutic mechanisms of licochalcone A (LCA), a bioactive flavonoid from licorice, against DN with emphasis on AGEs/RAGE signaling modulation. Male C57BL/6J mice were used to establish a high-fat diet/streptozotocin-induced DN model, followed by 4-week treatment with LCA (5, 10, 20 mg/kg/day) or metformin (250 mg/kg/day). LCA treatment significantly improved glucose tolerance, reduced fasting blood glucose levels, and ameliorated characteristic renal pathological changes including mesangial matrix expansion and collagen deposition. Mechanistically, LCA suppressed AGEs accumulation in both serum and renal tissues while downregulating RAGE expression, thereby attenuating NF- κ B-mediated inflammatory responses (TNF- α , IL-1 β , IL-6). Furthermore, LCA mitigated oxidative stress by restoring SOD activity and reducing MDA and ROS levels, and alleviated endoplasmic reticulum stress through suppression of the GRP78/PERK/ATF4/CHOP pathway, consequently reducing renal cell apoptosis. Serum metabolomics revealed that LCA corrected lipid metabolism disorders, with KEGG enrichment analysis highlighting ABC transporter pathway involvement. LCA treatment reversed diabetic dyslipidemia, prevented ectopic lipid accumulation in kidneys, and modulated the SREBP-1c/ABCA1 axis. These findings were validated in high glucose-treated HK-2 cells. In conclusion, LCA demonstrates multi-targeted nephroprotective effects in DN through suppression of AGEs/RAGE signaling and downstream modulation of inflammation, oxidative stress, endoplasmic reticulum stress, and lipid metabolism dysregulation.

Keywords: Diabetic nephropathy; Licochalcone A; Lipid metabolism; AGEs/RAGE; Inflammation; Endoplasmic reticulum stress

1. Introduction

Diabetic Nephropathy (DN) is one of the most serious microvascular complications of diabetes, characterized by progressive impairment of renal structure and function due to persistent hyperglycemia, and

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is closely linked to increased morbidity and mortality in diabetic patients[1]. Currently, the number of diabetes patients worldwide has reached 537 million and is expected to increase to 643 million by 2030. In China, the number of diabetes patients has exceeded 140 million, ranking first in the world, and it is expected to reach 174 million by 2045[2]. Approximately 20% to 40% of diabetic patients develop DN, which is not only a primary pathogenic factor for chronic kidney disease and end-stage renal failure but is also recognized as the leading cause of end-stage renal disease (ESRD)[3]. The pathogenesis of DN involves a complex interplay of metabolic, hemodynamic, and inflammatory mechanisms that collectively contribute to progressive renal dysfunction[4]. Therefore, exploring the pathogenesis and regulatory pathways of DN and identifying novel therapeutic targets and strategies have become an urgent priority.

DN is clinically characterized by progressive proteinuria and declining glomerular filtration rate[5], with pathological hallmarks including glomerular mesangial matrix expansion, tubulointerstitial fibrosis, basement membrane thickening, and altered creatinine clearance[6]. The formation of advanced glycation end products (AGEs) under hyperglycemic conditions represents a critical pathogenic mechanism in DN development[7]. These irreversible, cytotoxic compounds arise from non-enzymatic glycation reactions (Maillard reactions) between reducing sugars and proteins, lipids, or nucleic acids[8]. Upon accumulation in renal tissues, AGEs bind to their receptor for AGEs (RAGE), activating nuclear factor- κ B (NF- κ B) signaling pathways that trigger inflammatory cascades, leading to structural renal damage and functional impairment[9]. This process promotes reactive oxygen species generation, establishing oxidative stress that further induces endoplasmic reticulum (ER) stress[10,11]. Furthermore, abnormal AGEs accumulation facilitates low-density lipoprotein cholesterol (LDL-C) oxidation, contributing to lipid metabolism dysregulation—another fundamental aspect of DN pathophysiology[12,13]. This dysregulation typically manifests as dyslipidemia, characterized by elevated serum levels of triglycerides, total cholesterol, and LDL-C, coupled with decreased high-density lipoprotein cholesterol (HDL-C) concentrations[14]. Such metabolic perturbations promote ectopic lipid deposition within renal tissues, a phenomenon termed renal lipotoxicity, which directly impairs glomerular and tubular function[15]. Consequently, nutritional interventions targeting AGEs/RAGE reduction represent a promising therapeutic strategy for DN management.

Licochalcone A (LCA), one of the principal bioactive constituents of *Glycyrrhiza* species (Licorice), has been extensively utilized in food products, pharmaceuticals, nutraceuticals, and cosmetics, with its safety and efficacy well-established through traditional use and modern research[16]. LCA is a distinctive flavonoid compound isolated from licorice root extracts, characterized by its unique chalcone structure (3-dimethylallyl-4,4'-dihydroxy-6-methoxychalcone)[17], and exhibits diverse pharmacological activities including anti-inflammatory, antioxidant, antimicrobial, and antiviral properties[18]. In the context of metabolic disorders, LCA has demonstrated significant therapeutic potential by reducing body weight and decreasing inguinal and epididymal adipose tissue mass in high-fat diet (HFD)-induced obese mice, while simultaneously ameliorating hepatic steatosis, regulating serum triglycerides, low-density lipoprotein, and free fatty acid levels, and lowering fasting blood glucose concentrations[19,20]. Furthermore, existing studies

have demonstrated that LCA exerts therapeutic effects on DN through anti-inflammatory mechanisms[21]. However, the molecular targets and mechanistic pathways underlying LCA's renoprotective effects in DN remain incompletely elucidated. Whether LCA modulates the AGEs/RAGE signaling axis and how such modulation influences downstream pathological processes, including inflammation, oxidative stress, endoplasmic reticulum stress, and lipid metabolism dysregulation, have not been systematically investigated. Therefore, the present study aims to elucidate the therapeutic mechanisms of LCA in DN, with particular emphasis on its effects on AGEs/RAGE signaling and the subsequent multi-pathway renoprotective outcomes, thereby providing mechanistic insights that may guide future therapeutic strategies for DN management.

2. Material and Methods

2.1 Experimental design

Male C57BL/6J mice (6-8 weeks old, weighing 20-23 g) were purchased from Henan Silibase Biotechnology Co., Ltd. [production license: SCXK (Yu) 2020-0005] and housed under standard laboratory conditions (temperature: $25 \pm 2^{\circ}\text{C}$, humidity: 50-60%, 12-h light/dark cycle) with ad libitum access to food and water. After a 2-week acclimatization period, the DN model was established following previously described protocols[22,23]. Ten mice were maintained on standard chow diet as control group (Con), while the remaining animals received high-fat diet (60% fat, 20% carbohydrates, 20% protein) for 28 days. Subsequently, control mice received intraperitoneal injections of citrate buffer (pH 4.2), while high-fat diet-fed mice were administered Streptozocin (STZ, 50 mg/kg body weight, dissolved in citrate buffer) intraperitoneally once daily for 5 consecutive days following a 12-h fasting period. The establishment of DN model was validated by measuring fasting blood glucose (FBG) levels 5-7 days post-STZ injection; mice with fasting blood glucose ≥ 11.1 mmol/L were considered as successful model animals and randomly stratified into five groups (n=10 per group): the model group (Mod), low-dose LCA group (LCA-L, 5 mg/kg/day), medium-dose LCA group (LCA-M, 10 mg/kg/day), high-dose LCA group (LCA-H, 20 mg/kg/day), and the metformin (glucophage) group (MET, 250 mg kg⁻¹/day). All treatments were administered orally once daily for 4 weeks. After the treatment period, mice were fasted for 12 h before sacrifice, and blood samples were collected via cardiac puncture. Organs were harvested, weighed, and either fixed in 4% paraformaldehyde or snap-frozen in liquid nitrogen for subsequent analyses. The experimental protocol was approved by the Life Ethics Review Committee of the Henan Academy of Sciences (Ethics approval number: HNAS.EC202503a025).

2.2 Chemicals and reagents

LCA (Cat No. 58749-22-7, purity $\geq 98\%$) was purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). STZ (Cat No. MB12690) and metformin hydrochloride (Cat No. 1115-70-4) were obtained from Dalian Meilun Biotechnology Co., Ltd. (Dalian, China). Commercial assay kits for superoxide dismutase (SOD, Cat No. A001-3-2), malondialdehyde (MDA, Cat No. A003-1-2), and reactive oxygen

species (ROS, Cat No. G1746) were procured from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China) and Sevier Biotechnology Co., Ltd. (Wuhan, China), respectively. Primary antibodies against RAGE (Cat No. 16346-1-AP), ABCA1 (Cat No. 26564-1-AP), PERK (Cat No. 68482-1-Ig), phospho-PERK (Cat No. 82534-1-RR), ATF4 (Cat No. 10835-1-AP), CHOP (Cat No. 15204-1-AP), and glucose-regulated protein 78 kDa (GRP78, Cat No. 11587-1-AP) were obtained from Proteintech Group (Wuhan, China). SREBP-1 antibody (Cat No. Ab133125) was purchased from Abcam (Cambridge, UK). AGEs antibody (Cat No. AB_3103054) was sourced from MedChemExpress (Shanghai, China). Antibodies against NF- κ B p65 (Cat No. 8242S) and phospho-NF- κ B p65 (Cat No. 3033L) were obtained from Cell Signaling Technology (Danvers, MA, USA). All other chemicals and reagents were of analytical grade and purchased from standard commercial suppliers.

2.3 Biochemical analysis

The levels of advanced glycation end products (AGEs), TNF- α , IL-1 β and IL-6 in serum were quantified using enzyme-linked immunosorbent assay (ELISA) kits (Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) according to manufacturer's protocols. Serum lipid profiles including triglycerides, total cholesterol, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol were analyzed using a Chemray 800 automated biochemical analyzer (Rayto Life and Analytical Sciences Co., Ltd., Shenzhen, China). For oral glucose tolerance test (OGTT), mice were fasted for 12 h before oral administration of glucose solution (1 g/kg body weight). Blood glucose concentrations were measured at 0, 30, 60, 90, and 120 min using a glucometer, and the area under the curve was calculated. Renal tissue homogenates were prepared for assessment of oxidative stress markers including SOD activity, MDA content, and ROS levels using commercial assay kits following manufacturer's instructions.

2.4 Histological examination

Kidney tissues were fixed in 4% paraformaldehyde for 48 h, dehydrated through graded ethanol series, embedded in paraffin, and sectioned at 4 μ m thickness. Histomorphological changes were evaluated using hematoxylin and eosin (H&E) staining and periodic acid-Schiff (PAS) staining according to standard protocols[24]. Renal fibrosis was assessed by Masson's trichrome staining, while lipid accumulation was visualized using Oil Red O staining on frozen sections. Apoptotic cells in renal tissues were detected using a TUNEL assay kit (Servicebio, GDP1042) according to the manufacturer's instructions. For immunohistochemical analysis, paraffin sections were subjected to antigen retrieval, blocked with normal serum, and incubated with primary antibodies against AGEs (1:200) and RAGE (1:500) overnight at 4°C. Detection was performed using horseradish peroxidase-conjugated secondary antibodies. Digital images were captured using an automated quantitative pathological imaging system (Pannoramic MIDI, 3DHISTECH, Budapest, Hungary) and analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA).

2.5 Western blot

Renal tissues were lysed in RIPA buffer containing protease and phosphatase inhibitors. Protein concentrations were determined using the BCA protein assay kit. Equal amounts of protein (30–50 µg) were separated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes. After blocking with 5% nonfat milk, membranes were incubated with primary antibodies overnight at 4°C, followed by horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Protein bands were visualized using enhanced chemiluminescence reagent and quantified using TanonImage software (Tanon Science & Technology Co., Ltd., Shanghai, China). Protein expression levels were normalized to β-actin as the loading control.

2.6 Cell culture and in vitro studies

Human proximal tubular epithelial cells (HK-2) were cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C with 5% CO₂. To simulate hyperglycemic conditions, cells were treated with 30 mM high glucose[25]. Cells were divided into Con group, Mod group (high glucose treatment), and LCA treatment groups with varying concentrations (2.5, 5, and 10 µM) in the presence of high glucose. After 24 h treatment, cell viability was assessed using the Cell Counting Kit-8 assay. For immunofluorescence staining, cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 5% bovine serum albumin, and incubated with primary antibodies against AGEs and RAGE overnight at 4°C, followed by Alexa Fluor-conjugated secondary antibodies and nuclear counterstaining with DAPI.

2.7 Gene expression analysis

Total RNA was extracted from kidney tissues using the FastPure Complex Tissue/Cell Total RNA Isolation Kit (Vazyme Biotech, Nanjing, China). Complementary DNA was synthesized using the All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (TransGen Biotech, Beijing, China), and quantitative real-time PCR was performed using Green qPCR SuperMix (TransGen Biotech, Beijing, China) on a real-time PCR system. Gene expression levels were calculated using the $2^{(-\Delta\Delta Ct)}$ method with β-actin as the internal control.

2.8 Metabolomics analysis

Serum samples were analyzed using liquid chromatography-mass spectrometry for metabolomic profiling following established protocols[26]. Serum samples were deproteinized, and supernatants were subjected to LC-MS analysis. Differential metabolites between groups were identified based on fold change (≥ 1.5 or ≤ 0.67) and statistical significance ($P < 0.05$). Metabolic pathway analysis was conducted using the Kyoto Encyclopedia of Genes and Genomes database, and data analysis was performed using the GenesCloud platform (Personalbio, Shanghai, China).

2.9 Statistical analysis

All data were presented as mean ± standard. The graphical abstract was created using Figdraw. Statistical analyses were performed using GraphPad Prism 10.0 software (GraphPad Software, San Diego, CA, USA).

For multiple group comparisons, one-way analysis of variance followed by Dunnett's post hoc test was employed. Statistical significance was set at $P < 0.05$.

3. Results

3.1 Effects of LCA on blood glucose in DN mice

To investigate the therapeutic potential of LCA in DN progression, we established HFD/STZ-induced DN mice model according to previously validated protocols and implemented the experimental design depicted in Figure 1A[44]. Following DN induction, mice were randomly allocated into control (Con), model (Mod), and treatment groups. Baseline assessments revealed that FBG levels in both Mod and treatment groups were comparably elevated and significantly higher than those in the Con group, while body weights remained comparable across all experimental groups. Following 4 weeks of treatment intervention, LCA administration did not significantly alter body weight in DN mice (Figure 1B). However, FBG levels in the Mod group remained significantly elevated compared to the Con group, whereas treatment with all doses of LCA, as well as MET, resulted in marked reductions in FBG levels in DN mice (Figure 1C). Furthermore, OGTT analysis demonstrated that both LCA and MET treatment significantly improved systemic glucose tolerance compared to the Mod group, as evidenced by reduced area under the curve (AUC) values (Figure 1D) and improved glucose clearance kinetics over the 120-minute observation period (Figure 1E). Collectively, these findings demonstrate that LCA treatment effectively ameliorated glucose intolerance in DN mice, indicating its beneficial effects on glycemic control.

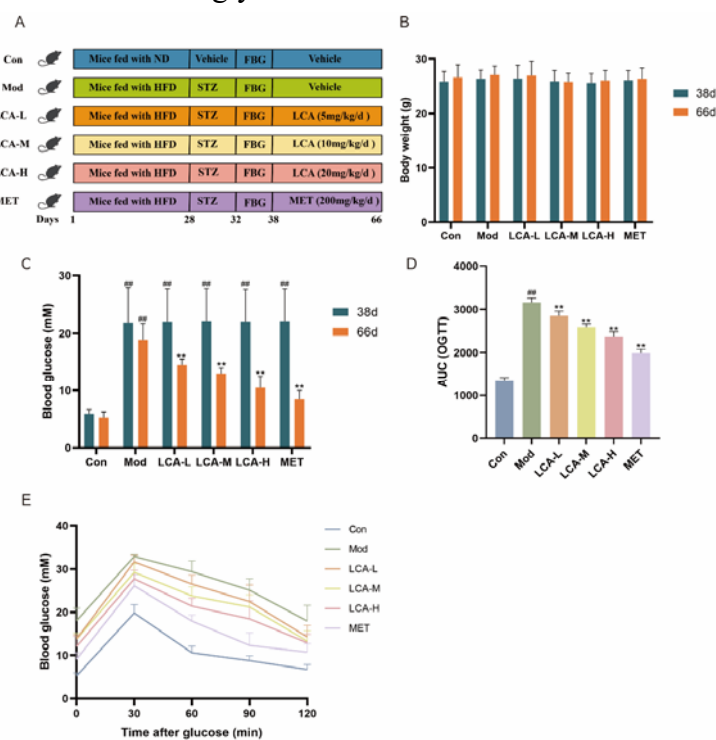


Figure. 1 LCA treatment ameliorates glucose intolerance in DN mice. (A) Experimental protocol. (B) Body weight changes. (C) The level of FBG. (D) OGTT area under curve analysis. (E) Glucose tolerance test profiles. Values are presented as means $\pm$ standard ($n=10$ per group). $^{(*)}P < 0.05$, $^{(**)}P < 0.01$. $^{\#}$ Compared to Con group. * Compared to Mod group.

3.2 Renoprotective effects of LCA against pathological injury in DN mice

Having established LCA's beneficial effects on glucose homeostasis, we next examined whether LCA could directly ameliorate renal pathological damage in DN mice. Histopathological analysis revealed that compared to the Con group, kidneys from the Mod group exhibited characteristic DN lesions, including disrupted glomerular architecture, mesangial matrix expansion, and pronounced glycated protein accumulation (Figure 2A). Meanwhile, the number of glomerular cells was significantly decreased and the glomerular area was increased in the Mod group (Figures 2B and 2C). Quantitative assessment confirmed significant increases in Masson-positive areas and PAS-positive regions in the Mod group compared to Con (Figure 2D and 2E). Treatment with LCA (all doses) and MET markedly attenuated these histopathological changes. These results suggest that LCA treatment exhibits renoprotective effects in DN mice.

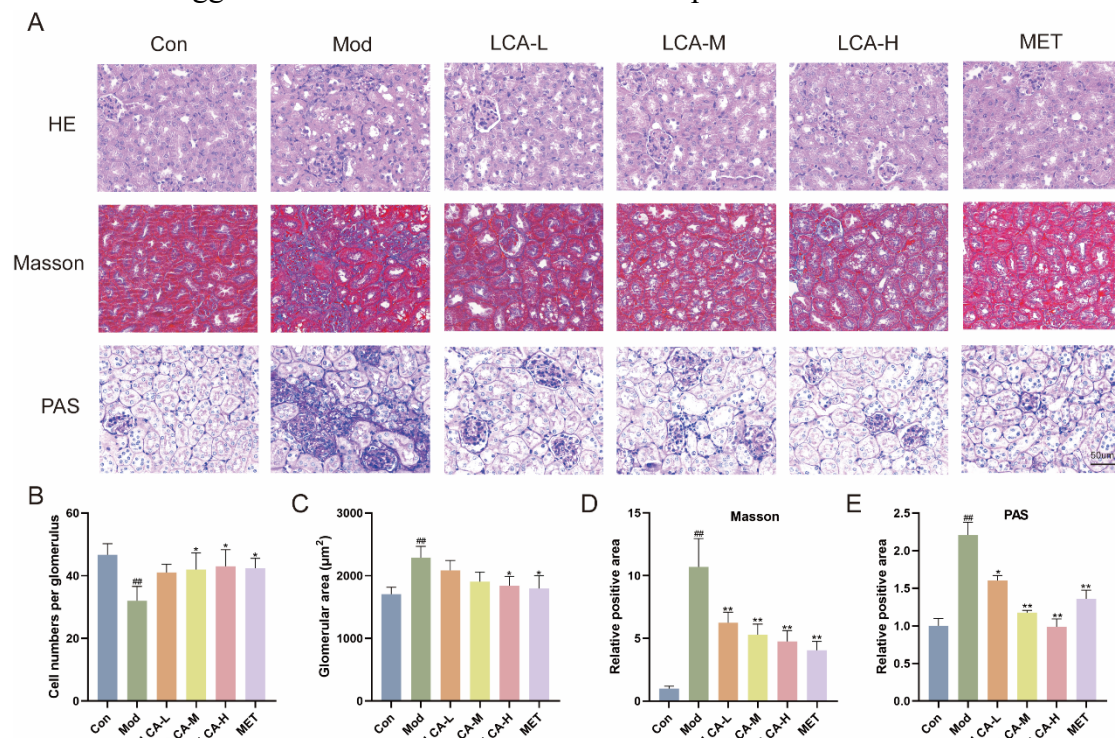


Figure. 2 LCA attenuates renal pathological damage in DN mice. (A) Representative photomicrographs of kidney sections stained with H&E, Masson's trichrome, and PAS (magnification: 20 $\times$). (B) Glomerular cell numbers. (C) Glomerular area. (D) Quantitative analysis of Masson's trichrome staining. (E) Quantitative analysis of PAS staining. Values are presented as means $\pm$ standard (n=5 per group). ^{##}(*) $P < 0.05$, ^{##}(**) $P < 0.01$. [#]Compared to Con group. ^{*}Compared to Mod group.

3.3 Effects of LCA on AGEs formation and the AGEs/RAGE signaling pathway

Following the observation that LCA exerted regulatory effects on blood glucose and protective effects on the kidneys, and given that hyperglycemia promotes AGEs formation, which subsequently triggers a cascade of reactions through RAGE, we investigated the role of the AGEs/RAGE axis in the pathogenesis of DN. Biochemical analysis revealed significantly elevated AGEs levels in both serum and kidney tissues of the Mod group compared to Con group, reflecting enhanced protein glycation under diabetic conditions, and treatment with LCA dose-dependently reduced both circulating and renal AGEs accumulation (Figure 3A and 3B). Immunohistochemical staining demonstrated intense AGEs and RAGE expression in DN kidneys (Figure 3E), and quantitative analysis confirmed significant upregulation of both AGEs and RAGE protein expression in the model group (Figure 3C and 3D). The effects of LCA on AGEs/RAGE were further validated at the

cellular level. Using a high glucose-induced HK-2 cell injury model, LCA treatment (0, 1.25, 2.5, 5, 10 μ M) showed no impact on HK-2 cell viability (Figure 3F). Therefore, we selected LCA (2.5, 5, 10 μ M) as the concentrations for subsequent experiments. Compared with the Con group, the viability of Mod group was significantly reduced, while LCA (LCA-L, LCA-M and LCA-H) treatment could increase cell viability (Figure 3G). Immunofluorescence results showed that the levels of AGEs and RAGE proteins in the cells of the Mod group were significantly higher than those in the Con group, while LCA (LCA-L, LCA-M and LCA-H) treatment could inhibit the expression of AGEs and RAGE proteins (Figure 3H-J). Therefore, LCA treatment suppressed AGEs accumulation and RAGE expression in renal tissues and kidney cells, thereby exerting renoprotective effects.

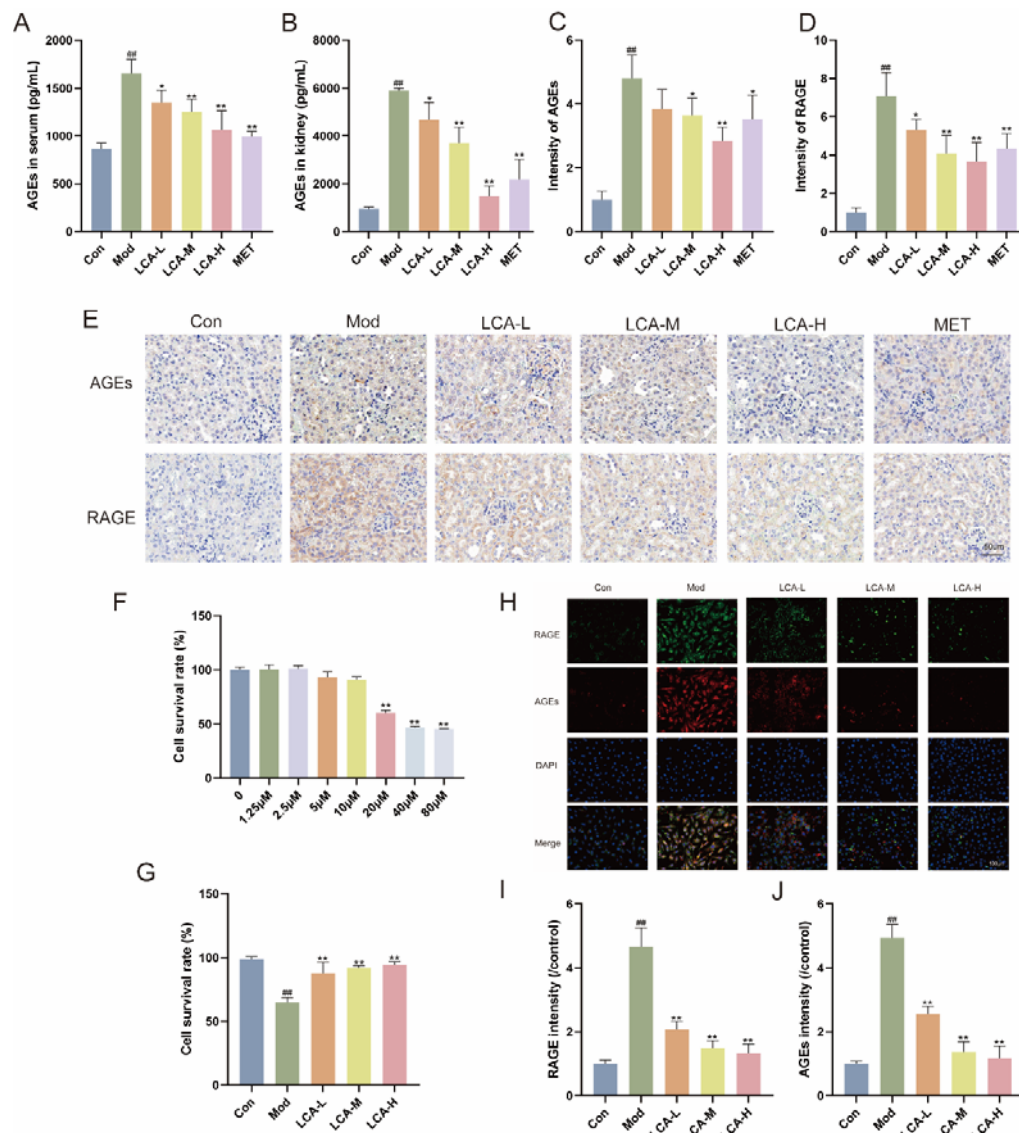


Figure 3 LCA inhibits AGEs formation and suppresses AGEs/RAGE signaling. (A-B) AGEs levels in serum and kidney tissues. (C-D) Quantitative analysis of AGEs and RAGE immunostaining intensity. (E) Representative immunohistochemical staining of AGEs and RAGE in kidney sections (magnification: 20 $\times$). (F) Cell viability of HK-2 cells treated with different concentrations of LCA assessed by CCK-8 assay. (G) Cell viability of HK-2 cells under HG conditions with LCA treatment. (H-J) Representative immunofluorescence images and quantitative analysis of AGEs and RAGE protein expression in HK-2 cells. Values are presented as means $\pm$ standard (n=5 per group). ^{*}(*) $P < 0.05$, ^{##}(**) $P < 0.01$. [#]Compared to Con group. ^{*}Compared to Mod group.

3.4 Effects of LCA on inflammatory response in DN

Studies have shown that AGEs binding to RAGE activates the downstream NF- κ B signaling pathway, thereby triggering an inflammatory cascade[27]. Therefore, we evaluated the levels of NF- κ B, a key inflammatory mediator, and its associated inflammatory cytokines. Analysis of inflammatory cytokines in mice serum demonstrated that TNF- α , IL-1 β , and IL-6 expression was significantly elevated in the Mod group compared with the Con group, indicating inflammatory activation during DN pathogenesis. Medium and high doses of LCA and MET reduced inflammatory cytokine expression to levels comparable with metformin treatment (Figures 4A-C). Consistent with these results, RT-qPCR analysis of renal tissues revealed significant upregulation of *TNF- α* , *IL-1 β* , and *IL-6* in the Mod group, which were attenuated to varying extents after treatment (Figures 4D-F). Western blot analysis revealed substantial NF- κ B upregulation in DN kidneys, which was significantly attenuated by LCA treatment in a dose-dependent manner (Figure 4G). Collectively, these findings indicate that LCA may attenuate inflammatory responses via suppression of the AGEs/RAGE signaling pathway and downstream regulation of NF- κ B expression.

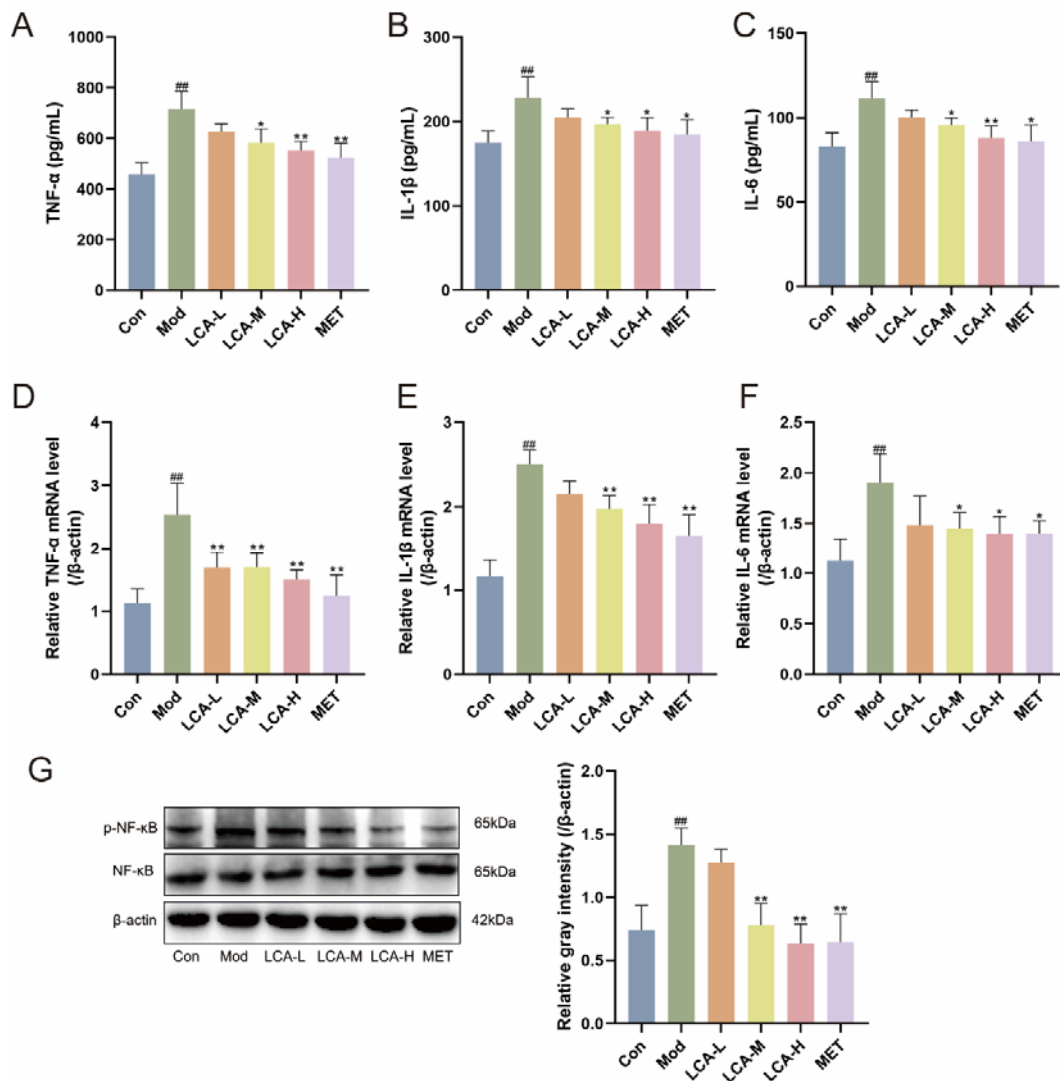


Figure. 4 LCA on inflammatory response in DN. (A-C) TNF- α , IL-1 β , and IL-6 level in serum. (D-F) Changes in *TNF- α* , *IL-1 β* , and *IL-6* mRNA expression levels. (G) Western blot analysis and quantification of NF- κ B proteins. Values are presented as means $\pm$ standard (n=5 per group). [#](*) P < 0.05, ^{##}(**) P < 0.01. [#]Compared to Con group. *Compared to Mod group. (F) Western blot analysis and quantification of NF- κ B protein expression.

3.5 Effects of LCA on oxidative stress

Previous studies have demonstrated that AGEs-RAGE interaction triggers oxidative stress, thereby aggravating diabetes and its complications[28]. We therefore examined the effects of LCA on oxidative stress in DN. Compared with the Con group, renal tissues from Mod group mice exhibited significantly elevated levels of ROS and MDA, accompanied by markedly reduced SOD activity (Figures 5A-C). LCA treatment dose-dependently alleviated oxidative stress, as demonstrated by decreased ROS and MDA levels and restoration of SOD activity. These findings were further validated in high glucose-induced HK-2 cells (Figures 5D-F). Collectively, these results suggest that LCA may mitigate oxidative stress in DN via suppression of the AGEs/RAGE signaling pathway.

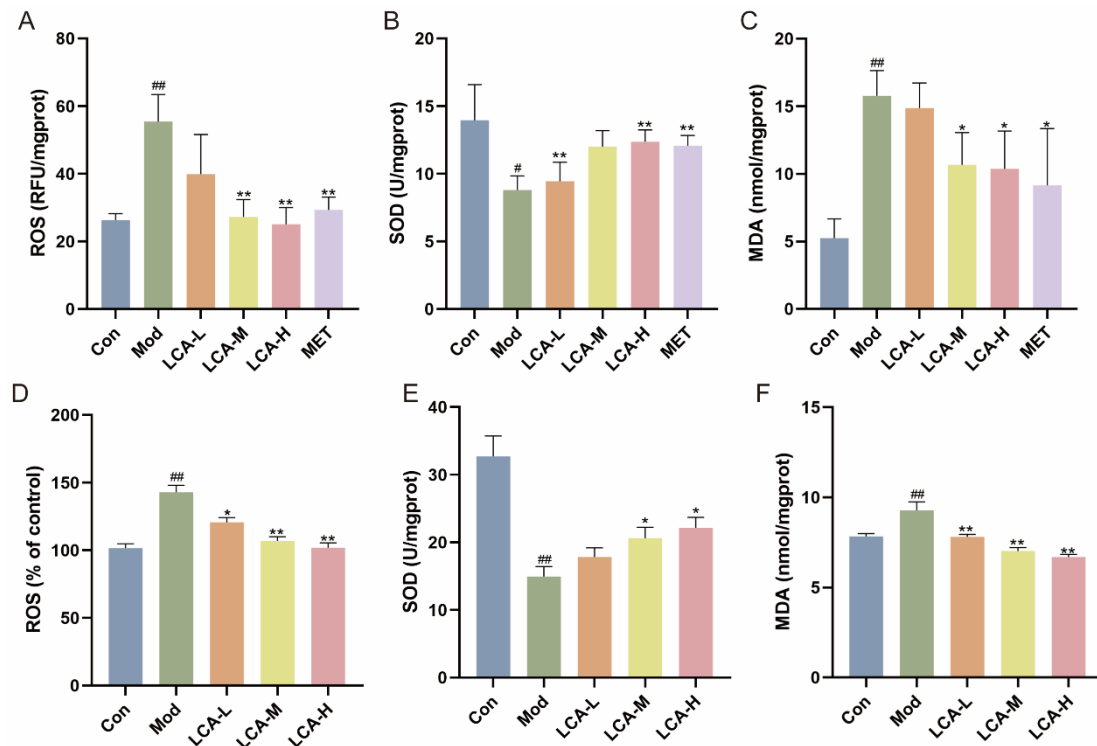


Figure. 5 LCA reduces oxidative stress. (A-C) Levels of ROS, SOD activity, and MDA content in renal tissues. (D-F) Oxidative stress markers including ROS, SOD activity, and MDA levels in HK-2 cells. Values are presented as means $\pm$ standard (n=5 per group). [#](*) $P < 0.05$, ^{##}(**) $P < 0.01$. [#]Compared to Con group. *Compared to Mod group.

3.6 Effects of LCA on endoplasmic reticulum stress and apoptosis

In DN, there exists a reciprocal relationship between ER stress and oxidative stress, which promotes disease onset and progression[29]. Under the influence of hyperglycemia, an ER stress inducer, accumulation of unfolded or misfolded proteins occurs in the ER lumen, further promoting cell apoptosis[30]. Western blot analysis revealed that the ER stress markers GRP78, phosphorylated PERK (p-PERK), ATF4, and CHOP were significantly upregulated in renal tissues of Mod group mice, and LCA treatment dose-dependently suppressed the expression of these ER stress markers (Figure 6A). Meanwhile, following high glucose treatment of HK-2 cells, the mRNA levels of *GRP78*, *ATF4*, and *CHOP* were significantly elevated in the Mod group compared with the Con group, which were reduced by LCA treatment (Figure 6B-D). TUNEL staining further revealed marked apoptosis in both mouse renal tissues and cells from the Mod group, which was ameliorated to varying extents by LCA treatment at different doses (Figure 6E and 6F). Therefore, LCA

may exert indirect cytoprotective effects by inhibiting the ER stress-mediated GRP78/PERK/ATF4/CHOP pathway, thereby reducing apoptosis and conferring renoprotective effects.

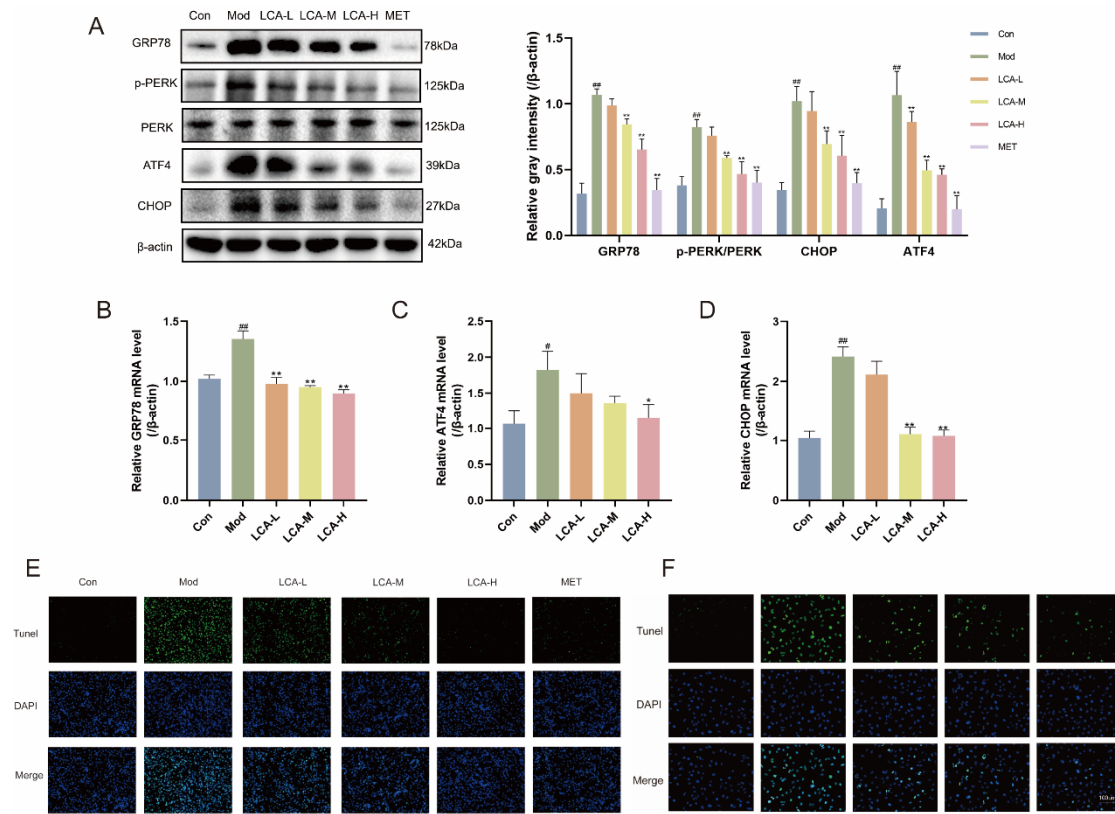


Figure 6 The effects of LCA on ER stress and apoptosis. (A) Western blot analysis and quantification of ER stress pathway proteins. (B) The mRNA expression levels of *GRP78*. (C) The mRNA expression levels of *ATF4*. (D) The mRNA expression levels of *CHOP*. (E) Apoptosis of mouse kidney tissue cells. (F) LCA treatment induces apoptosis of HK-2 cells under HG conditions. Values are presented as means $\pm$ standard ($n=5$ per group). #(*) $P < 0.05$, ##(**) $P < 0.01$. #Compared to Con group. *Compared to Mod group.

3.7 Effects of LCA on dyslipidemia and renal lipid accumulation in DN mice

Dyslipidemia and ectopic lipid deposition represent key pathogenic factors driving DN progression, and amelioration of lipid metabolism can attenuate tubular cell apoptosis through modulation of ER stress[31]. Therefore, we further investigated the effects of LCA on blood lipids and lipid accumulation in DN mice. Serum lipid profile analysis revealed that the Mod group exhibited characteristic diabetic dyslipidemia, including significantly elevated levels of TC, TG, and LDL-C, alongside reduced HDL-C compared to Con group. Treatment with LCA at all doses, as well as MET, significantly ameliorated these lipid abnormalities (Figure 7A-C, E). Oil Red O staining of renal tissues revealed marked lipid accumulation in the glomerular and tubular structures of the model group, which was significantly reduced by both LCA and MET treatment, with the most pronounced effect observed at high-dose LCA (Figure 7D). These results indicate that LCA effectively ameliorates dyslipidemia and prevents renal lipid accumulation, thereby exerting renoprotective effects.

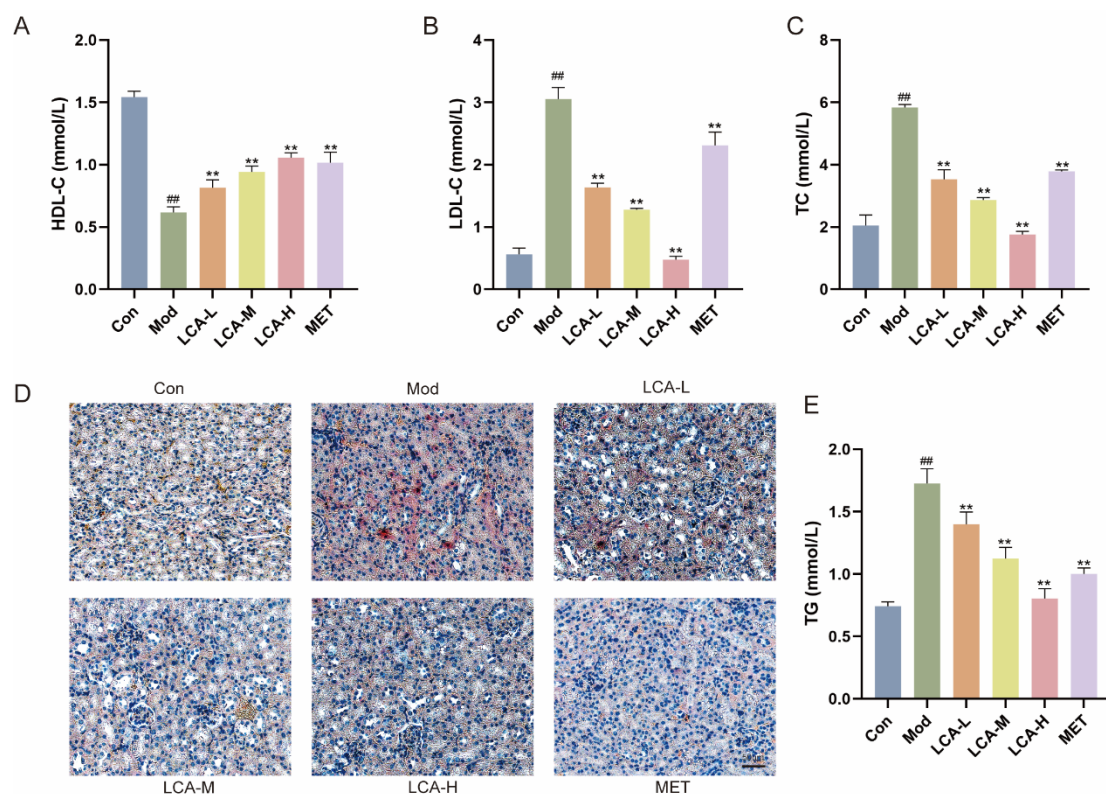


Figure 7 LCA improves lipid metabolism and reduces renal lipid accumulation in DN mice. (A) The HDL-C level in serum. (B) The LDL-C level in serum. (C) The TG level in serum. (D) Representative Oil Red O staining of renal tissues (magnification: 20 $\times$). (E) The TG level in serum. Values are presented as means $\pm$ standard (n=5 per group). [#](*) P < 0.05, ^{##}(**) P < 0.01. [#]Compared to Con group. *Compared to Mod group.

3.8 Regulatory effects of LCA on the lipid metabolic profile in DN mice

To further investigate the alterations in metabolite levels during LCA-regulated lipid deposition and whether they are associated with AGEs/RAGE modulation, a non-targeted metabolomics approach was employed to systematically analyze the serum metabolic profiles of DN mice. The results showed that the QC samples exhibited highly overlapped TIC and were tightly clustered in the PCA model, indicating the stability of the instrument during analysis and the reliability of the obtained data (Figure 8A). Clear separation was observed between Con and Mod groups, with LCA treatment groups (LCA-L, LCA-M, and LCA-H) forming distinct clusters separate from the model group, indicating dose-dependent metabolic restoration (Figure 8B and 8C). Differential metabolite screening using OPLS-DA with criteria of VIP > 1.0 and P < 0.05 identified 358 significantly altered metabolites between control and model groups. The OPLS-DA model was utilized to identify differential metabolites among groups, and the permutation test plot demonstrated good explanatory and predictive characteristics (Figure 8D). Metabolites were primarily enriched in lipids and lipid-like molecules as well as organic acids and derivatives (Figure 8E). Further analysis of lipid metabolism-related differential metabolites revealed that high-dose LCA treatment reversed the changes in 68 metabolites, of which 17 belonged to the lipid class and exhibited profiles similar to the control group (Figure 8F and Table 1). KEGG enrichment analysis indicated that the ATP-binding cassette (ABC) transporter pathway was one of the major metabolic pathways affected by LCA treatment in DN mice (Figure 8G). ABCA1 in the ABC transporter pathway plays a crucial role in cellular cholesterol efflux and lipid homeostasis, while sterol regulatory element-binding protein-1C (SREBP-1C), which acts upstream of ABCA1, can be regulated by the

AGEs/RAGE axis and plays an important role in lipid metabolism[32–34]. Therefore, Western blot analysis of mouse renal tissues revealed that ABCA1 expression was significantly decreased while SREBP-1C expression was markedly upregulated in the Mod group. LCA treatment dose-dependently restored the expression of these related proteins, with the most pronounced effects observed at medium and high doses (Figure 8J). Consistent with these findings, the in vitro cell model showed that *ABCA1* mRNA expression was significantly reduced and *SREBP* was elevated in the Mod group, which were reversed following drug administration (Figures 8H and 8I). Collectively, these results suggest that LCA may modulate lipid metabolism and alleviate lipid accumulation in DN mice by influencing the ABC transporter pathway through the AGEs/RAGE signaling pathway.

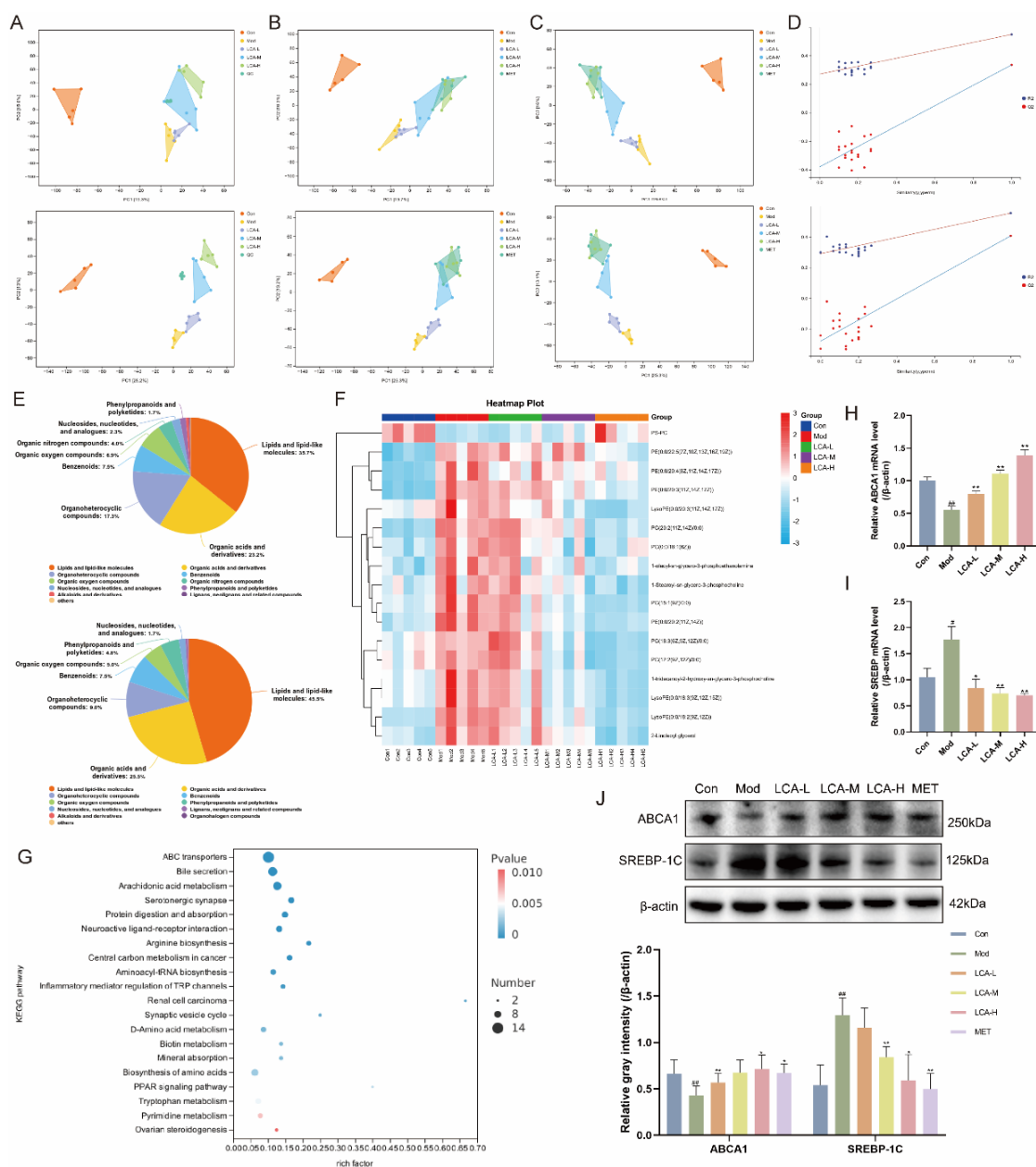


Figure 8 The effects of LCA in lipid metabolism. (A) PCA score plots with QC. (B–C) PCA and PLS-DA score plots demonstrating group separation. (D) Permutation test plot. (E) Total differential metabolites in positive and negative ion modes. (F) Heatmap and hierarchical clustering of differential lipid metabolites. (G) KEGG pathway enrichment analysis of top 20 affected pathways. (H) The mRNA expression levels of *ABCA1*. (I) The mRNA expression levels of *SREBP*. (J) Western blot analysis and quantification of ABCA1 and SREBP-1C protein expression. Values are presented as means $\pm$ standard (n=5 per group). #(* $P < 0.05$, ##(** $P < 0.01$). #Compared to Con group. *Compared to Mod group.

Table 1. 17 kinds of differential lipid metabolites.

NO .	Formula	Metabolites	Delta (ppm)	Change trend (Con/Mod)	Change trend (Mod/LC A-L)	Change trend (Mod/LC A-M)	Change trend (Mod/LC A-H)
1	C27H46NO7P	PE(0:0/22:5(7Z,10Z, 13Z,16Z,19Z))	-3.1	↑***	—	—	↓**
2	C25H44NO7P	PE(0:0/20:4(8Z,11Z, 14Z,17Z))	-3.27	↑**	—	—	↓*
3	C25H46NO7P	PE(0:0/20:3(11Z,14 Z,17Z))	-2.88	↑*	—	↓*	↓***
4	C25H46NO7P	LysoPE(0:0/20:3(11 Z,14Z,17Z))	-2.73	↑*	—	↓*	↓***
5	C28H54NO7P	PC(20:2(11Z,14Z)/0: 0)	-2.86	↑*	—	—	↓***
6	C26H52NO7P	PC(0:0/18:1(6Z))	-3.28	↑**	—	↓*	↓*
7	C23H46NO7P	1-oleoyl-sn-glycero- 3-phosphoethanolam ine	-3.38	↑**	—	↓*	↓**
8	C26H54NO7P	1-Stearoyl-sn-glycer o-3-phosphocholine	-3.16	↑*	—	—	↓**
9	C23H46NO7P	PC(15:1(9Z)/0:0)	-3.14	↑**	—	↓**	↓***
10	C25H48NO7P	PE(0:0/20:2(11Z,14 Z))	-2.59	↑***	—	↓**	↓***
11	C26H48NO7P	PC(18:3(6Z,9Z,12Z) /0:0)	-3.03	↑*	—	↓*	↓***
12	C25H48NO7P	PC(17:2(9Z,12Z)/0:0)	-3.07	↑*	—	↓*	↓**
13	C21H44NO7P	1-tridecanoyl-2-hydr oxy-sn-glycero-3-ph osphocholine	2.71	↑**	—	↓**	↓***
14	C23H42NO7P	LysoPE(0:0/18:3(9Z, 12Z,15Z))	-1.93	↑**	—	↓**	↓***
15	C23H44NO7P	LysoPE(0:0/18:2(9Z, 12Z))	-3.3	↑***	—	↓**	↓***
16	C21H38O4	2-Linoleoyl glycerol	-3.44	↑***	—	↓*	↓***
17	C28H54NO10P	PS-PC	-2.82	↑**	—	—	↓**

4. Discussion

Restoration of glucose homeostasis represents a fundamental therapeutic objective in DN management[35]. In the present study, LCA treatment significantly improved glucose tolerance and reduced FBG levels in DN mice without affecting body weight, indicating that its metabolic benefits are attributable to direct glucose-lowering effects rather than appetite suppression. This glucose-lowering capacity of LCA is relevant to DN pathogenesis, as improved glycemic control directly reduces the formation of AGEs, which are key mediators of DN[36]. The accumulation of AGEs in renal tissues, particularly within glomeruli, triggers a cascade of pathological events that are central to DN progression[37]. AGEs-rich glomerular environments promote the recruitment of inflammatory monocytes, which subsequently release pro-inflammatory cytokines, thereby initiating and perpetuating the inflammatory responses that are intimately associated with DN advancement and renal injury[38–40]. Our findings demonstrated that LCA treatment significantly reduced the expression of major pro-inflammatory cytokines including TNF- α , IL-1 β , and IL-6 in the blood and renal tissues, while simultaneously ameliorating characteristic histopathological

features such as mesangial matrix expansion and collagen deposition. These observations are consistent with previous studies demonstrating the anti-inflammatory properties of LCA[21]. RAGE serves as a critical signal transduction receptor for AGEs and plays a pivotal role in DN pathogenesis[41]. Upon AGEs binding, RAGE activates the transcription factor nuclear factor- κ B (NF- κ B), which subsequently promotes the expression of downstream pro-inflammatory genes[7]. Our results revealed that LCA effectively inhibited AGE formation in both serum and renal tissues, while concurrently reducing RAGE expression and suppressing downstream NF- κ B activation.

Furthermore, the AGEs/RAGE signaling axis plays a crucial role in orchestrating oxidative stress and subsequent ER stress during DN progression[42]. Upon binding to RAGE, AGEs trigger the activation of NADPH oxidase and mitochondrial dysfunction, leading to excessive generation of ROS that overwhelm cellular antioxidant defense systems[43]. This oxidative imbalance disrupts normal cellular homeostasis and creates a pathological environment that promotes ER stress[44]. The accumulated ROS directly damage ER membranes and impair protein folding machinery, while simultaneously depleting cellular antioxidant enzymes such as SOD, further exacerbating oxidative damage[45]. In our study, LCA treatment significantly improved SOD activity and reduced MDA levels in both renal tissues and cells. Persistent oxidative stress triggers the unfolded protein response (UPR) by activating key ER stress sensors, with glucose-regulated protein 78 kDa (GRP78) serving as the master regulator of ER homeostasis and acting as a molecular chaperone to assist protein folding under stress conditions[46]. However, when ER stress becomes overwhelming, GRP78 dissociates from PERK, leading to PERK activation and subsequent phosphorylation, which promotes ATF4 translation and ultimately results in CHOP upregulation and cellular apoptosis[47]. Our findings demonstrated that LCA effectively suppressed the expression of ER stress markers in the GRP78/PERK/ATF4/CHOP pathway in diabetic kidneys, thereby influencing renal cell apoptosis.

DN is commonly associated with lipid metabolism disorders, with dyslipidemia occurring throughout all disease stages[48]. Ectopic lipid deposition refers to the excessive accumulation of lipids, particularly triglycerides (TGs), in non-adipose tissues such as kidneys, liver, heart, and pancreas, leading to lipotoxic injury and a cascade of pathophysiological changes[49,50]. Our results demonstrated that DN mice exhibited significantly elevated levels of TG, TC, and LDL-C, while HDL-C levels were markedly decreased. Metabolomic analysis further revealed profound disruptions in lipid metabolism in DN mice. Following LCA treatment, these lipid abnormalities were effectively reversed, thereby alleviating tissue injury. Further metabolomic analysis revealed that LCA's inhibitory effects on lipid deposition in DN mice may be closely associated with ABC transporters. ABCA1, a member of the ABC transporter family, plays a crucial role in the development and progression of DN. ABCA1 deficiency increases intracellular cholesterol accumulation, leading to inflammation and endoplasmic reticulum stress, ultimately promoting cellular apoptosis[51]. Conversely, ABCA1 upregulation promotes cholesterol efflux, suppresses endoplasmic reticulum stress, and ameliorates glomerular injury and early DN[52,53]. Sterol regulatory

element-binding proteins (SREBPs) regulate low-density lipoprotein receptors and fatty acid uptake, contributing to excessive lipid deposition in DN kidneys[54]. SREBP-1c overexpression leads to increased de novo lipogenesis, lipid accumulation, and insulin resistance[55]. Studies have shown that reducing SREBP-1c expression can increase HDL-C levels, thereby promoting ABCA1-mediated cholesterol efflux and reducing cholesterol accumulation[56]. Furthermore, the RAGE has been shown to activate the SREBP pathway, triggering a vicious cycle of lipid accumulation, inflammation, and tissue injury that culminates in glomerular sclerosis, tubulointerstitial fibrosis, and DN progression[42]. Our findings demonstrated that LCA treatment significantly reduced SREBP-1c expression while simultaneously upregulating ABCA1 expression in both renal tissues and high glucose-treated HK-2 cells, effects that correlated with suppression of AGEs accumulation and RAGE expression. These results suggest that LCA's regulation of the SREBP-1c/ABCA1 axis may be mediated, at least in part, through inhibition of the AGEs/RAGE signaling pathway. This represents a novel mechanistic link between AGEs/RAGE signaling and lipid dysregulation in DN pathogenesis. It should be noted that while our metabolomic and biochemical findings suggest a connection between LCA's effects on AGEs/RAGE and lipid metabolism regulation, the precise molecular mechanisms warrant further investigation.

5. Conclusions

This study establishes LCA as a multi-targeted nephroprotective agent in DN through suppression of the AGEs/RAGE signaling pathway. Using integrated in vivo and in vitro approaches, we demonstrate that LCA inhibits AGEs accumulation and RAGE expression, thereby disrupting a central pathogenic axis that drives DN progression. By intervening in the AGEs/RAGE signaling pathway, LCA produces coordinated therapeutic effects including attenuation of NF- κ B-mediated inflammatory responses, mitigation of oxidative stress, suppression of ER stress through the GRP78/PERK/ATF4/CHOP pathway with subsequent reduction in renal cell apoptosis, and prevention of ectopic lipid deposition via modulation of the SREBP-1c/ABCA1 axis, thereby interrupting the pathological cycle linking lipid accumulation to ER stress and cellular injury. These findings establish LCA as a promising multi-targeted therapeutic candidate for DN that addresses complex and interconnected pathological mechanisms through suppression of AGEs/RAGE signaling.

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Declaration of competing interest

The authors declare no competing financial interests or personal relationships that could have influenced the work reported in this study.

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