

Original Article

Low-protein calorie-restricted diet attenuates renal injury and facilitates podocyte autophagy in type 2 diabetic rats

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Abstract: Caloric restriction has been shown to exert renoprotective effects in diabetic rats. Dietary protein restriction appears to slow the progression of nephropathy in diabetic patients. However, very little is known about the effects of a low-protein calorie-restricted (LPCR) diet on diabetes-related renal injury, particularly podocyte injury. The present study investigated the effects of an LPCR diet on renal injury and podocyte autophagy in type 2 diabetic rats. Rats were randomly divided into three feeding groups: normal protein (NP, 20% protein), low protein (LP, 8% protein), and LPCR (8% protein, 30% restriction). After 4 weeks of dietary intervention, urine albumin-to-creatinine ratios, creatinine clearance, serum creatinine, kidney-to-body weight ratios, renal histology, podocyte density, and glomerular autophagy levels, as well as glomerular basement membrane thickness and the number of autophagic vacuoles in podocytes were evaluated. Results showed that the LPCR diet attenuated renal injury, including podocyte injury, in type 2 diabetic rats. Moreover, the reduced number of autophagic vacuoles in podocytes and insufficient podocyte autophagy observed in the diabetic rats were reversed after feeding with the LPCR diet. These findings suggest that an LPCR diet can ameliorate renal injury in type 2 diabetic rats. These effects appear to be linked to the activation of podocyte autophagy via regulation of mTOR/AMPK signaling pathways. Taken together, this study links LPCR, renal injury, and podocyte autophagy while highlighting potential therapeutic roles for LPCR in the treatment of diabetes-related renal injuries.

Keywords: Diabetic nephropathy, low-protein diet, caloric restriction, podocytes, autophagy

Introduction

Diabetic nephropathy (DN), the leading cause of end-stage renal disease, has become a serious health concern worldwide. Furthermore, the increasing prevalence of type 2 diabetes necessitates identification of effective therapeutic approaches directed at preventing the development and progression of diabetic kidney disease. DN is characterized by a gradual increase in urinary albumin excretion, mainly attributed to disturbances in the glomerular filtration membrane [1, 2]. Notably, podocytes play a prominent role in maintaining the integrity of the glomerular filtration barrier [3], while

decreased podocyte number per glomerulus and foot process effacement are crucial events in the early stages of DN [4, 5]. Podocytes are terminally differentiated cells with a limited capacity for cell division and regeneration. Thus, self-repair mechanisms are vital to maintaining podocyte homeostasis [6].

Autophagy, an evolutionarily conserved homeostatic cellular process, has been shown to be involved in multiple physiological activities, playing a key role in many diseases [7, 8]. Podocytes, for example, have a high basal level of autophagy [9, 10]. A growing body of evidence suggests that impaired autophagy par-

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Table 1. Composition of experimental diets

Ingredient (% w/w)	High-fat diet	Normal-protein diet	Low-protein diet
Casein	23.00	22.60	9.10
Cornstarch	6.00	37.15	50.65
Maltodextrin	11.25	13.20	13.20
Sucrose	20.00	10.00	10.00
Cellulose	5.80	5.00	5.00
Soybean oil	3.00	7.00	7.00
Lard	24.00	0.00	0.00
Mineral Mix	5.21	3.50	3.50
Vitamin Mix	1.16	1.00	1.00
Choline bitartrate	0.23	0.25	0.25
L-Cystine	0.35	0.30	0.30

icipates in DN pathogenesis. Indeed, insufficient podocyte autophagy appears to be involved in the pathogenesis of podocyte injury and loss, leading to albuminuria in DN [11]. Therefore, activation of autophagy in podocytes could be used as a potential therapy to prevent development and progression of DN [12].

Caloric restriction, which can activate autophagy, has been shown to exert renoprotective effects in diabetic rats [13, 14]. Moreover, dietary protein restriction appears to slow the progression of nephropathy in diabetic patients [15, 16]. However, the effects of a low-protein calorie-restricted (LPCR) diet on type 2 diabetes-related renal injury remain largely unknown, particularly regarding the role of autophagy activation in podocytes.

In this study, the effects of an LPCR diet on renal injury and podocyte autophagy were investigated in rats with type 2 diabetes, induced with a combination of high-fat diet and low-dose streptozotocin (STZ).

Materials and methods

Animals and diets

Male Sprague-Dawley (SD) rats, approximately 6 weeks old and weighing 160-180 g, were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China, Permit No. SCXK (jing) 2012-0001). Rats were housed in cages in a temperature-controlled (24±2°C) room with a 12-hour light/dark cycle. They were allowed free access to water and food. All animal protocols were approved by the

Institutional Animal Care and Use Committee of Nanjing University of Chinese Medicine. All diets were purchased from Beijing Keao Xieli Feed Co., Ltd. (Beijing, China). The high-fat diet consisted of 52% fat, 17% protein, 31% carbohydrate, and 4.7 kcal/g energy. The normal-protein diet consisted of 64% carbohydrate, 16% fat, and 20% protein, while the low-protein diet contained 76% carbohydrate, 16% fat, and 8% protein. Casein was the major source of dietary protein and the two diets were

isocaloric (3.8 kcal/g). Compositions of these experimental diets are listed in **Table 1**.

Induction of type 2 diabetes and experimental design

After being acclimatized to the housing conditions for one week, rats were fed a high-fat diet for 5 weeks. They were then injected intraperitoneally with 35 mg/kg of STZ (Sigma-Aldrich Co., St Louis, MO, USA) dissolved in 0.1 M citrate buffer (pH 4.4). Fasting blood glucose levels were detected at days 7 and 14. Rats with fasting blood glucose levels greater than or equal to 11.1 mmol/L were included in this study. The diabetic rats were randomized into 3 groups of ten each: normal protein (NP, 20% protein), low protein (LP, 8% protein), and low-protein calorie-restricted (LPCR, 8% protein, 30% restriction). In addition, ten non-diabetic SD rats served as the normal control (NC) group. Food intake was measured daily. Body weights and fasting blood glucose levels were measured before and at the end of dietary intervention in all four groups. After 4 weeks of dietary intervention, 8-hour urine samples were collected using individual metabolic cages. Blood samples were obtained from the retro-orbital plexus and serum was collected by centrifugation at 4,000 rpm for 15 minutes at 4°C. Samples were stored at -80°C until analysis. Rats were anesthetized with 10% chloral hydrate (0.3 mL/100 g) by intraperitoneal injection. The left kidney was removed, decapsulated, and weighed. A small portion of the renal cortex was instantly fixed in 5% glutaraldehyde for electron microscopic examination, while the remaining kidney tissue was

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cross-sectioned and fixed in 10% formalin for histopathological examination. The right kidney was immediately removed for the isolation of glomeruli.

Blood and urinary analysis

Blood glucose concentrations were detected with a glucometer (Yuwell, Danyang, China). Levels of serum total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), serum creatinine, and urinary creatinine were measured with a Cobas 8000 automatic biochemical analyzer (Roche, Mannheim, Germany), according to standard laboratory procedures. Creatinine clearance (Ccr) was calculated with the standard formula and adjusted by 100 g/rat weight [17, 18]. Concentrations of serum insulin were determined with a rat insulin enzyme-linked immunosorbent assay (ELISA) kit (American Laboratory Products Co., Windham, NH, USA). The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated as the fasting insulin ($\mu\text{U/mL}$) \times fasting glucose (mmol/L)/22.5. Homeostasis model assessment of beta cell function (HOMA- β) was calculated as the fasting insulin \times 20/(fasting glucose -3.5). Urinary albumin concentration was assayed using a rat microalbuminuria ELISA kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

Histopathology and immunohistochemistry

Cross-sections of the kidneys (3 μm thick) were prepared and stained with hematoxylin and eosin (HE), as well as periodic acid-Schiff reagent (PAS). After measuring the glomerular areas with Image-Pro Plus 6.0 software, glomerular volumes were calculated using the Weibel and Gomez formula [19], whereby glomerular volume (V_g) = $\text{area}^{1.5} \times 1.38/1.01$ (1.38: shape coefficient, 1.01: size distribution coefficient). To evaluate mesangial matrix expansion, 15 glomeruli were randomly selected from each rat and the PAS-positive material in the mesangial area and glomerular tuft area were measured using Image-Pro Plus 6.0 software.

Paraffin-embedded kidney sections (2 μm) were immunostained using the peroxidase-based EnVision technique. Briefly, tissue sections were incubated with a rabbit polyclonal

anti-Wilms' tumor 1 (WT1) antibody (1:400; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. The reaction was then developed with a horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (DAKO, Glostrup, Denmark) for 30 minutes at room temperature (20-25°C). This was followed by diaminobenzidine staining and counterstaining with hematoxylin. Number of podocytes was estimated by counting the number of WT1-positive cells per glomerular tuft area in 15 randomly selected glomeruli.

Ultrastructural examination

Ultrathin sections of renal cortex were obtained and stained with uranyl acetate and lead citrate, followed by examination with a JEM-1010 electron microscope (JEOL, Tokyo, Japan). Glomerular basement membrane (GBM) thickness was measured with Image-Pro Plus 6.0 software and the mean GBM thickness was calculated for 20 randomly selected fields from each rat. Furthermore, the average number of autophagic vacuoles in podocytes was calculated at 40,000 \times magnification from 15 randomly selected fields per section.

Isolation of glomeruli

Glomeruli were isolated using standard sieving techniques, as described previously [20]. Briefly, the renal cortex was separated and cut into small fragments. They were then passed through a sequence of stainless steel screens with 80-, 150-, and 200-mesh sizes. The glomeruli were collected by centrifuging samples from the 200-mesh sieve at 2,000 rpm for 5 minutes at 4°C and stored at -80°C until use.

Glomerular protein extraction and Western blot analysis

Protein was isolated from glomeruli using radioimmunoprecipitation assay buffer containing 1 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined by bicinchoninic acid protein assay (Biotime, Shanghai, China). Equal amounts of protein were loaded into the wells of SDS-PAGE gels and electrophoretically transferred onto polyvinylidene difluoride membranes. Membranes were blocked in PBST containing 5% skimmed milk for 2 hours, followed by incubation with primary antibodies against microtubule-ass-

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Table 2. Effects of an LPCR diet on body weights and fasting blood glucose levels in diabetic rats

Group	Body weight (g)		Weight change	Glucose (mmol/L)		Glucose change
	0 w	4 w		0 w	4 w	
NC	263±8	314±9	51±7	6.30±0.39	9.01±0.36	2.71±0.57
NP	308±16	286±19	-21±5 ^b	16.58±1.78 ^a	26.03±1.68 ^b	9.45±2.75
LP	292±14	263±21	-29±8 ^b	15.96±1.40 ^a	18.30±2.86	2.34±2.35
LPCR	302±18	241±27	-61±15 ^{b,c}	17.30±2.12 ^a	9.68±1.57 ^d	-7.62±1.50 ^d

Differences before and after dietary intervention (0 and 4 week) in diabetic rats. Data are presented as mean ± SEM, n = 5-8 in each group. ^aP < 0.05 vs. NC group; ^bP < 0.01 vs. NC group; ^cP < 0.05 vs. NP group; ^dP < 0.01 vs. NP group.

Table 3. Effects of an LPCR diet on physical and biochemical parameters in diabetic rats

Parameter	NC	NP	LP	LPCR
Serum creatinine (umol/L)	23.83±2.01	18.13±1.11	22.40±2.40	16.67±2.28
Ccr/100 g (mL/min)	0.55±0.06	0.75±0.10	0.50±0.04	0.57±0.12
TG (mmol/L)	0.77±0.09	0.88±0.08	0.49±0.15	0.44±0.12 ^c
TC (mmol/L)	2.15±0.14	1.60±0.12	1.70±0.29	1.85±0.26
LDL-C (mmol/L)	0.67±0.03	0.45±0.07	0.50±0.12	0.41±0.06
HDL-C (mmol/L)	1.62±0.09	1.13±0.14	1.41±0.22	1.55±0.22
HOMA-IR	3.05±0.40	5.64±0.82 ^b	3.32±0.33 ^c	1.69±0.32 ^d
HOMA-β	27.63±2.37	3.84±0.51 ^b	6.89±1.56 ^a	16.05±3.88
Kidney weight/body weight (g/kg)	5.55±0.23	7.31±0.36 ^b	6.99±0.32 ^a	6.31±0.52
Urine albumin/creatinine (mg/mmol)	28.31±5.36	106.20±7.58 ^a	96.84±15.56	27.76±3.09 ^c

Data are presented as mean ± SEM, n = 5-8 in each group. ^aP < 0.05 vs. NC group; ^bP < 0.01 vs. NC group; ^cP < 0.05 vs. NP group; ^dP < 0.01 vs. NP group.

ociated protein light chain 3 (LC3) (1:3,000), mechanistic target of rapamycin (mTOR) (1:3,000), phosphorylated mTOR (p-mTOR) (1:4,000), AMP-activated protein kinase (AMPK) (1:2,000), or phosphorylated AMPK (p-AMPK) (1:3,000) overnight at 4°C. These membranes were then incubated with the appropriate HRP-linked anti-rabbit secondary antibody at room temperature for 2 hours. Protein bands were detected using an enhanced chemiluminescence kit (Millipore, Billerica, MA, USA). The density of each protein band was quantified by Quantity One 4.6.2 software. β-actin was used as a loading control and detected with an anti-β-actin antibody purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA).

Statistical analyses

Data are presented as mean ± SEM. GraphPad Prism 5 was used for all statistical analyses. Bartlett's test of equality of variances and Kolmogorov-Smirnov tests were used to evaluate variance and normality of the data, respectively. Comparisons were made using one-way

ANOVA, followed by Tukey-Kramer multiple comparisons test. When data was not normally distributed or the variance was not equal, comparisons were conducted using non-parametric Kruskal-Wallis tests, followed by Dunn's multiple comparisons test. *P* < 0.05 indicates statistical significance.

Results

Physical and biochemical effects of an LPCR diet in diabetic rats

Table 2 shows changes in body weights and fasting blood glucose levels for different groups before and after dietary intervention. Mean body weight was decreased in all diabetic rats by the end of the experiment, with a significant decrease observed in the LPCR group compared to that in the NP group (*P* < 0.05). Regarding levels of fasting blood glucose, only the LPCR group showed a significant decrease compared to the NP group at the end of dietary intervention (*P* < 0.01).

As shown in **Table 3**, HOMA-IR, kidney-to-body weight ratios, and microalbuminuria (urine

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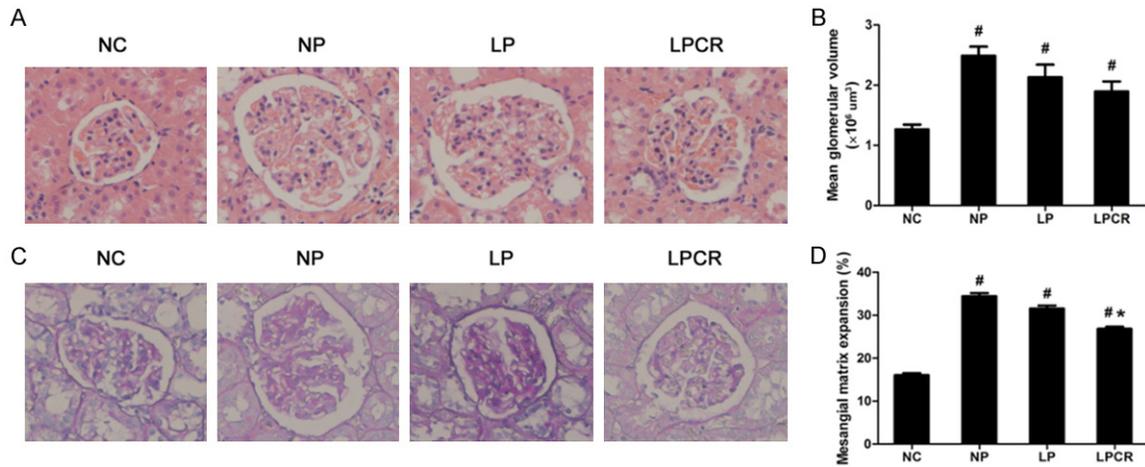


Figure 1. Effects of an LPCR diet on renal histological injury in diabetic rats. A: Representative photomicrographs of HE-stained kidney sections. Original magnification, $\times 400$. B: Quantitative analysis of the mean glomerular volume (at least 20 randomly selected tissue sections from each group). Data are mean \pm SEM ($n = 4$, $^{\#}P < 0.05$ vs. NC group). C: Representative photomicrographs of PAS-stained kidney sections. Original magnification, $\times 400$. D: Quantitative analysis of the mesangial matrix area. Data are mean \pm SEM ($n = 4$, $^{\#}P < 0.05$ vs. NC group, $^{*}P < 0.05$ vs. NP group).

albumin: creatinine ratios) were significantly higher in the NP group than the NC group, while HOMA- β was significantly lower in the NP group than the NC group. No significant differences were noted in serum creatinine and creatinine clearance of diabetic rats compared to those of non-diabetic rats. Treatment with an LPCR diet for 4 weeks induced a significant reduction in HOMA-IR and microalbuminuria, compared to the NP group. HOMA- β and kidney-to-body weight ratios in the LPCR group were also ameliorated, but to a lesser extent. Concerning serum lipid profiles, the LPCR group had significantly lower levels of TG compared to the NP group. However, there were no statistically significant differences in TC, LDL-C, or HDL-C.

Histological effects of an LPCR diet on renal injury in diabetic rats

Compared to non-diabetic rats, a remarkable increase was noted in the mean glomerular volume in diabetic rats. Although the mean glomerular volume in the LPCR group was lower than that in the NP and LP groups, the difference was not statistically significant. Furthermore, the mesangial matrix was more extensive in the glomeruli of diabetic rats than in non-diabetic rats, but treatment with an LPCR diet reduced this expansion (**Figure 1**).

An LPCR diet attenuates podocyte injury in diabetic rats

Podocyte density was examined by immunohistochemical staining of WT1. The number of podocytes per glomerular tuft area was significantly lower in diabetic rats than in non-diabetic rats. In contrast, a remarkable increase in podocyte density was noted in diabetic rats after treatment with an LPCR diet (**Figure 2A** and **2B**). Ultrastructural examinations revealed pronounced podocyte foot process fusion and GBM thickening in diabetic rats. Compared to the NP group, thickness of the GBM was significantly reduced and podocyte injury was attenuated in the LPCR group, whereas no evident changes were observed in the LP group (**Figure 2C** and **2D**).

An LPCR diet increases autophagic vacuole number in podocytes and autophagy levels in the glomeruli of diabetic rats

The number of autophagic vacuoles in podocytes detected by electron microscopy in the NP and LP groups was significantly decreased compared to the NC group. However, a significantly higher number of autophagic vacuoles were detected in podocytes in the LPCR group compared to the NP group (**Figure 3A** and **3B**). As demonstrated by Western blot analysis, expression of LC3-II/LC3-I in the glomeruli of

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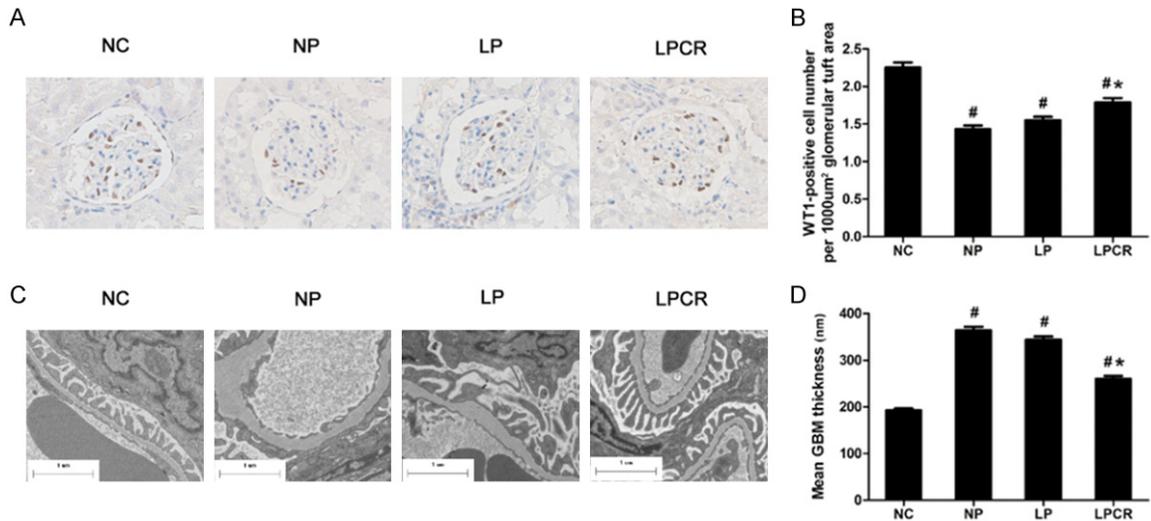


Figure 2. Effects of an LPCR diet on podocyte density and injury in diabetic rats. A: Representative glomeruli immunostained with WT1 (podocyte marker). Original magnification, $\times 400$. B: Quantitative analysis of the podocyte density. Data are mean \pm SEM ($n = 4$, $\#P < 0.05$ vs. NC group, $*P < 0.05$ vs. NP group). C: Representative electron micrographs of glomerular filtration barrier. Scale bar, 1 μ m. D: Quantitative analysis of the mean GBM thickness. Data are mean \pm SEM ($n = 3$, $\#P < 0.05$ vs. NC group, $*P < 0.05$ vs. NP group).

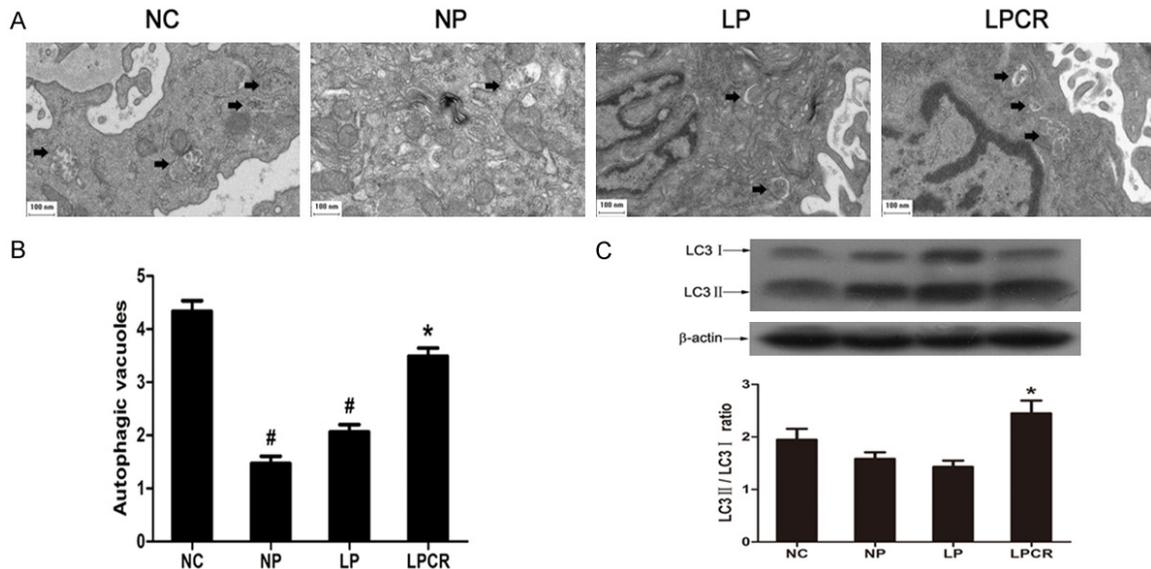


Figure 3. Effects of an LPCR diet on autophagic vacuoles number in podocytes and autophagy levels in glomeruli of diabetic rats. A: Representative electron micrographs of autophagic vacuoles (black arrows) in podocytes. Scale bar, 100 nm. B: Quantitative analysis of autophagic vacuoles in podocytes. Data are mean \pm SEM ($n = 3$, $\#P < 0.05$ vs. NC group, $*P < 0.05$ vs. NP group). C: Representative Western blot and densitometric analysis of LC3-II/LC3-I expression in isolated glomeruli. Data are mean \pm SEM ($n = 5$, $*P < 0.05$ vs. NP group).

diabetic rats was lower than that in non-diabetic rats. However, this difference was not statistically significant. Notably, a remarkable upregulation of LC3-II/LC3-I was observed in the glomeruli of diabetic rats fed an LPCR diet (Figure 3C).

AMPK/mTOR pathways activated in the glomeruli of diabetic rats fed an LPCR diet

Expression of AMPK and mTOR in the glomerulus were determined by Western blot analysis. As shown in Figure 4, the ratio of pAMPK/AMPK

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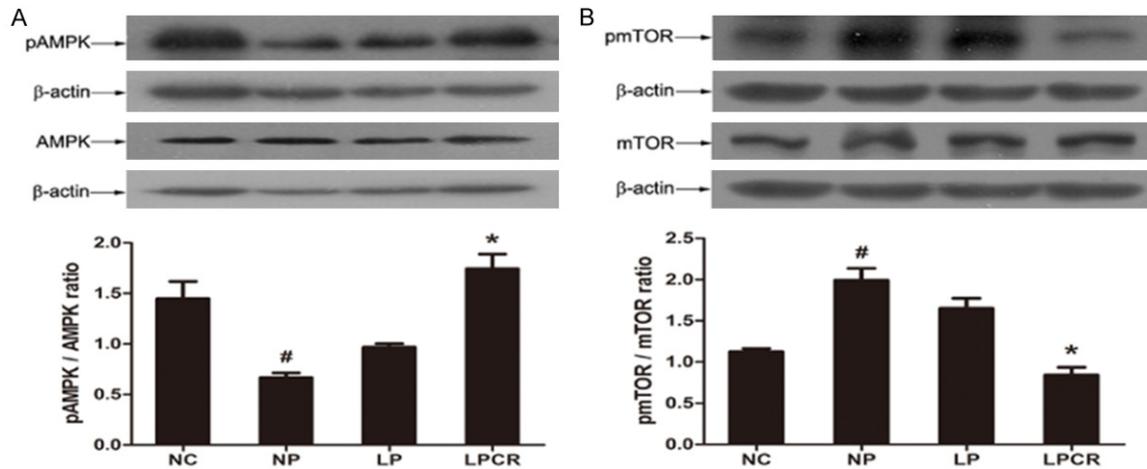


Figure 4. Effects of an LPCR diet on AMPK and mTOR expression in glomeruli of diabetic rats. Representative Western blot and densitometric analysis of pAMPK/AMPK (A) and pmTOR/mTOR (B) expression in isolated glomeruli. Data are mean \pm SEM ($n = 5$, [#] $P < 0.05$ vs. NC group, ^{*} $P < 0.05$ vs. NP group).

was significantly lower in the NP group than in the NC group, while the ratio of pmTOR/mTOR was significantly elevated. However, treatment with an LPCR diet for 4 weeks induced remarkable upregulation of pAMPK/AMPK and significant downregulation of pmTOR/mTOR, compared to the NP group.

Discussion

Caloric restriction, also known as dietary restriction, is defined as a reduction in food intake without malnutrition. It has established that caloric restriction can extend lifespans and attenuate age-related degeneration and diseases [21, 22]. Animal and human studies have shown that caloric restriction exerts renoprotective effects in diabetes [13, 14, 23]. Thus, a low-protein diet is recommended for patients with early-stage DN, according to clinical guidelines established by the American Diabetes Association [24]. Meta-analyses have shown that dietary protein restriction appears to slow the progression of nephropathy in diabetic patients [15, 16]. However, the effects of an LPCR diet on renal injury, particularly podocyte injury, in type 2 diabetes remain unexplored. Principal results of the present study are as follows: (a) Diabetic rats fed an LPCR diet exhibited reduced body weight, serum triglycerides, and fasting blood glucose levels, as well as improved insulin resistance compared to diabetic rats fed an NP diet; (b) This LPCR diet attenuated renal injury, including podocyte injury, in type 2 diabetic rats; and (c) The reduced

number of autophagic vacuoles in podocytes and insufficient podocyte autophagy observed in the diabetic rats were reversed after feeding with an LPCR diet.

Effects of an LPCR diet on renal function and histology were explored in rats with type 2 diabetes, induced by the combination of a high-fat diet and low-dose STZ intraperitoneal injection. These diabetic rats developed features of early-stage DN, as shown by microalbuminuria, elevated kidney-to-body weight ratios, accumulation of mesangial matrix, and diffuse thickening of the GBM. Treatment with an LPCR diet led to a significant decrease in microalbuminuria, mesangial matrix expansion, and GBM thickness. In addition, present results show that elevated fasting blood glucose and HOMA-IR in diabetic rats were significantly ameliorated by the LPCR diet. Regarding changes in body weights and serum lipid profiles, the LPCR group showed a significant reduction in body weight and TG compared to the NP group. These results suggest that an LPCR diet attenuates renal injury and improves glucose and lipid metabolism in type 2 diabetic rats.

Podocyte injury plays a vital role in the development of DN. A reduction in podocyte number and density has been linked to albuminuria and progression of DN [4, 25]. The present study used WT1 as a podocyte marker to calculate the podocyte number, showing that the decreased number of podocytes per glomerular tuft area in the diabetic rats could be improved

by intake of an LPCR diet. Additionally, pronounced podocyte foot process fusion was observed by electron microscopy in diabetic rats, a condition that was inhibited after being fed an LPCR diet. These data suggest that renoprotective effects of an LPCR diet in type 2 diabetic rats might be linked to improvement of podocyte injuries.

Autophagy is a highly regulated lysosomal protein degradation pathway that eliminates protein aggregates and damaged organelles, having a crucial impact on cellular homeostasis. Accumulating evidence has demonstrated that impaired autophagy is involved in DN pathogenesis. Calorie restriction and its mimetics, notably rapamycin and resveratrol, which can induce autophagy, have been shown to exert renoprotective effects and improve dysregulated autophagy in DN rodent models [13, 26, 27]. As high levels of autophagy observed in the glomerulus can be attributed to podocytes [9], this study measured expression of LC3 protein in the glomerulus lysate as a measurement of autophagy in the podocytes. It was found that the LPCR diet increased the ratio of LC3-II/LC3-I, a marker of autophagy, in the glomeruli of diabetic rats. Moreover, the reduced number of autophagic vacuoles, including both autophagosomes and autolysosomes, in podocytes was improved significantly following treatment with the LPCR diet. Present data suggests that an LPCR diet may attenuate podocyte injuries by facilitating podocyte autophagy.

Moreover, mTOR and AMPK pathways, two major nutrient-sensing signaling cascades, regulate autophagy in opposite ways [6, 7, 28]. As the major negative regulator of autophagy, mTOR signaling plays a pivotal role in podocyte homeostasis. Inhibition of this pathway can protect podocytes and prevent progressive DN [27, 29]. In contrast, AMPK signaling is a potent positive regulator of autophagy. Its activation restores autophagic activity in diabetic kidneys [6]. Furthermore, AMPK pathways can cross-talk with mTOR signaling to inhibit mTOR activity. Present results showed decreased expression of pmTOR/mTOR and increased expression of pAMPK/AMPK in the glomeruli of diabetic rats after being fed an LPCR diet, suggesting that AMPK/mTOR pathways may be involved in the LPCR-induced activation of autophagy in this animal model.

The present study also investigated the effects of an LP diet on renal function and histology in type 2 diabetic rats. Diabetic rats fed the LP diet for 4 weeks showed a mild, but not significant, decrease in microalbuminuria, mesangial matrix expansion, GBM thickness, and kidney-to-body weight ratios, as well as mean glomerular volume. More recently, Kitada et al. [30] found that a very low-protein diet improved advanced diabetic renal injury by restoring autophagy in type 2 diabetic rats. The discrepancy between this previous investigation and the current study may be due to the relatively insufficient protein restriction and short experimental period used here.

In conclusion, this study demonstrates the beneficial effects of an LPCR diet on renal injury, including podocyte injury, in type 2 diabetic rats. An LPCR diet appears to reduce podocyte injury by activating podocyte autophagy via regulation of mTOR/AMPK signaling pathways. Taken together, these data indicate that an LPCR diet may have the potential to prevent and/or treat early DN. While further studies are required to elucidate the full mechanisms of these observed renoprotective effects, the present study highlights potential therapeutic roles for LPCR in the treatment of diabetes-related renal injuries.

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Disclosure of conflict of interest

None.

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References

- [1] Gross JL, de Azevedo MJ, Silveiro SP, Canani LH, Caramori ML, Zelmanovitz T. Diabetic nephropathy: diagnosis, prevention, and treatment. *Diabetes Care* 2005; 28: 164-176.
- [2] Jefferson JA, Shankland SJ, Pichler RH. Proteinuria in diabetic kidney disease: a mechanistic viewpoint. *Kidney Int* 2008; 74: 22-36.
- [3] Brinkkoetter PT, Ising C, Benzing T. The role of the podocyte in albumin filtration. *Nat Rev Nephrol* 2013; 9: 328-336.
- [4] Pagtalunan ME, Miller PL, Jumping-Eagle S, Nelson RG, Myers BD, Rennke HG, Coplon NS, Sun L, Meyer TW. Podocyte loss and progressive glomerular injury in type II diabetes. *J Clin Invest* 1997; 99: 342-348.
- [5] Stitt-Cavanagh E, MacLeod L, Kennedy C. The podocyte in diabetic kidney disease. *ScientificWorldJournal* 2009; 9: 1127-1139.
- [6] Ding Y, Choi ME. Autophagy in diabetic nephropathy. *J Endocrinol* 2015; 224: R15-30.
- [7] Boya P, Reggiori F, Codogno P. Emerging regulation and functions of autophagy. *Nat Cell Biol* 2013; 15: 713-720.
- [8] Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. *J Pathol* 2010; 221: 3-12.
- [9] Hartleben B, Godel M, Meyer-Schwesinger C, Liu S, Ulrich S, Kobler S, Wiech T, Grahammer F, Arnold SJ, Lindenmeyer MT, Cohen CD, Pavenstadt H, Kerjaschki D, Mizushima N, Shaw AS, Walz G, Huber TB. Autophagy influences glomerular disease susceptibility and maintains podocyte homeostasis in aging mice. *J Clin Invest* 2010; 120: 1084-1096.
- [10] Fang L, Zhou Y, Cao H, Wen P, Jiang L, He W, Dai C, Yang J. Autophagy attenuates diabetic glomerular damage through protection of hyperglycemia-induced podocyte injury. *PLoS One* 2013; 8: e60546.
- [11] Yasuda-Yamahara M, Kume S, Tagawa A, Maegawa H, Uzu T. Emerging role of podocyte autophagy in the progression of diabetic nephropathy. *Autophagy* 2015; 11: 2385-2386.
- [12] Liu N, Xu L, Shi Y, Zhuang S. Podocyte autophagy: a potential therapeutic target to prevent the progression of diabetic nephropathy. *J Diabetes Res* 2017; 2017: 3560238.
- [13] Kitada M, Takeda A, Nagai T, Ito H, Kanasaki K, Koya D. Dietary restriction ameliorates diabetic nephropathy through anti-inflammatory effects and regulation of the autophagy via restoration of Sirt1 in diabetic Wistar fatty (fa/fa) rats: a model of type 2 diabetes. *Exp Diabetes Res* 2011; 2011: 908185.
- [14] Tikoo K, Tripathi DN, Kabra DG, Sharma V, Gai kwad AB. Intermittent fasting prevents the progression of type I diabetic nephropathy in rats and changes the expression of Sir2 and p53. *FEBS Lett* 2007; 581: 1071-1078.
- [15] Nezu U, Kamiyama H, Kondo Y, Sakuma M, Morimoto T, Ueda S. Effect of low-protein diet on kidney function in diabetic nephropathy: meta-analysis of randomised controlled trials. *BMJ Open* 2013; 3.
- [16] Pedrini MT, Levey AS, Lau J, Chalmers TC, Wang PH. The effect of dietary protein restriction on the progression of diabetic and nondiabetic renal diseases: a meta-analysis. *Ann Intern Med* 1996; 124: 627-632.
- [17] Arreola-Mendoza L, Reyes JL, Melendez E, Martin D, Namorado MC, Sanchez E, Del Razo LM. Alpha-tocopherol protects against the renal damage caused by potassium dichromate. *Toxicology* 2006; 218: 237-246.
- [18] Dezoti Fonseca C, Watanabe M, Vattimo Mde F. Role of heme oxygenase-1 in polymyxin B-induced nephrotoxicity in rats. *Antimicrob Agents Chemother* 2012; 56: 5082-5087.
- [19] Lane PH, Steffes MW, Mauer SM. Estimation of glomerular volume: a comparison of four methods. *Kidney Int* 1992; 41: 1085-1089.
- [20] Savin VJ, Terreros DA. Filtration in single isolated mammalian glomeruli. *Kidney Int* 1981; 20: 188-197.
- [21] Fontana L, Partridge L, Longo VD. Extending healthy life span—from yeast to humans. *Science* 2010; 328: 321-326.
- [22] Liu C, Li C, Chen J, Liu Y, Cheng Q, Xiang X, Chen G. Effects of a very low-calorie diet on insulin sensitivity and insulin secretion in overweight/obese and lean type 2 diabetes patients. *Diabetes Metab* 2015; 41: 513-515.
- [23] Giordani I, Malandrucchio I, Donno S, Picconi F, Di Giacinto P, Di Flaviani A, Chioma L, Frontoni S. Acute caloric restriction improves glomerular filtration rate in patients with morbid obesity and type 2 diabetes. *Diabetes Metab* 2014; 40: 158-160.
- [24] American Diabetes Association. 9. Microvascular complications and foot care. *Diabetes Care* 2016; 39 Suppl 1: S72-80.
- [25] Kalani A, Mohan A, Godbole MM, Bhatia E, Gupta A, Sharma RK, Tiwari S. Wilm's tumor-1 protein levels in urinary exosomes from diabetic patients with or without proteinuria. *PLoS One* 2013; 8: e60177.
- [26] Huang SS, Ding DF, Chen S, Dong CL, Ye XL, Yuan YG, Feng YM, You N, Xu JR, Miao H, You Q, Lu X, Lu YB. Resveratrol protects podocytes against apoptosis via stimulation of autophagy in a mouse model of diabetic nephropathy. *Sci Rep* 2017; 7: 45692.
- [27] Xiao T, Guan X, Nie L, Wang S, Sun L, He T, Huang Y, Zhang J, Yang K, Wang J, Zhao J. Rapamycin promotes podocyte autophagy and

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- ameliorates renal injury in diabetic mice. *Mol Cell Biochem* 2014; 394: 145-154.
- [28] Kim J, Kundu M, Viollet B, Guan KL. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nat Cell Biol* 2011; 13: 132-141.
- [29] Godel M, Hartleben B, Herbach N, Liu S, Zschiedrich S, Lu S, Debreczeni-Mor A, Lindemeyer MT, Rastaldi MP, Hartleben G, Wiech T, Forni A, Nelson RG, Kretzler M, Wanke R, Pavenstadt H, Kerjaschki D, Cohen CD, Hall MN, Ruegg MA, Inoki K, Walz G, Huber TB. Role of mTOR in podocyte function and diabetic nephropathy in humans and mice. *J Clin Invest* 2011; 121: 2197-2209.
- [30] Kitada M, Ogura Y, Suzuki T, Sen S, Lee SM, Kanasaki K, Kume S, Koya D. A very-low-protein diet ameliorates advanced diabetic nephropathy through autophagy induction by suppression of the mTORC1 pathway in Wistar fatty rats, an animal model of type 2 diabetes and obesity. *Diabetologia* 2016; 59: 1307-1317.