Original Article
Renoprotective effects of L-Carnitine in streptozotocin-induced diabetic nephropathy

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Abstract: *Aim:* L-Carnitine (LC) exerts protective effects on various types of injuries. This study aimed to investigate whether LC treatment would confer renoprotection in a rat model of streptozotocin (STZ)-induced diabetic nephropathy (DN). *Methods:* Diabetes was established by intraperitoneal STZ injection (65 mg/kg) in Sprague Dawley rats. Diabetic rats were treated daily for 12 weeks with LC (50 or 200 mg/kg/d intravenously). The effects of LC on STZ-induced DN were evaluated by assessing renal function, urinary protein excretion, histopathology, macrophage infiltration, expression of proinflammatory and prosclerotic cytokines, apoptotic cell death, oxidative stress, and expression of nuclear factor kappa B (NF-κB). *Results:* LC administration significantly decreased glomerulosclerosis, preserved the number of podocytes, and reduced macrophage infiltration, which accompanied with improvements in urinary protein excretion and renal dysfunction. At the molecular level, LC treatment suppressed expression of proinflammatory and prosclerotic cytokines, which paralleled by significant attenuation of NF-κB expression. LC treatment also attenuated increase of apoptotic cell death and oxidative stress. *Conclusion:* LC has a renoprotective effect on STZ-induced DN.

Keywords: L-Carnitine, diabetic nephropathy, oxidative stress, apoptosis, nuclear factor kappa B

Introduction

Diabetes mellitus (DM) is the leading cause of end-stage renal disease (ESRD) worldwide. Despite strict control of glycemic and metabolic abnormalities, the percentage of diabetic patients entering ESRD because of DM remains as high as 40% [1, 2]. Diabetic nephropathy (DN) is a common and serious complication of DM requiring costly renal-replacement therapies such as dialysis or kidney transplantation. Therefore, minimizing incidence of DN may improve quality of life among people with DM and may reduce the overall burden on society. DN is characterized pathologically by glomerular inflammation, mesangial proliferation, and progressive glomerulosclerosis, eventually leading to proteinuria and renal failure [3]. Molecular mechanisms underlying the evolution of DN are extremely complex, and several mediators have been implicated. Of these, oxi-dative stress, chemoattractants, fibrotic cyto-kines, and apoptosis have been proposed as important players [4-6].

L-Carnitine (LC, β-hydroxy-γ-N-trimethyl ammo-nium-butyrate), a quaternary ammonium compound, is a cofactor required for the transport of long-chain fatty acids into the mitochondria for energy production in peripheral tissues. LC inhibits free radical generation, helping to prevent impairment of fatty acid beta-oxidation in mitochondria and protecting tissues from damage by repairing oxidized membrane lipids [7]. LC has also been reported to be a direct scavenger of O_2^- and H_2O_2 [8]. There is overwhelming evidence that, through its antioxidative effects, LC supplementation plays a beneficial role in protecting against insulin resistance, diabetic podocyte injury, and diabetes-induced endo-thelial dysfunction [9, 10]. We have previously demonstrated that LC treatment protects

against cyclosporine A (CsA)-induced pancreatic and renal injury in rats [11]. The renoprotective effects of LC are also mirrored in prevention of ischemia-reperfusion injury [12], glycerol- and contrast-induced nephropathy [13, 14], hypertension-associated renal fibrosis [15, 16], cisplatin nephropathy [17], and doxorubicin-induced nephrotic syndrome [18]. However, its role in DN remains unexplored.

Considering the above mentioned findings, we hypothesized that LC would confer renoprotection against DN. To test this hypothesis, we investigated the effects of LC in a rat model of streptozotocin (STZ)-induced DN.

Materials and methods

Experiment and treatment schedule

Male Sprague Dawley rats (Charles River, Technology, Korea) weighting 240-260 g were housed in individual cages in a temperature- and light-controlled environment and allowed free access to tap water and standard laboratory chow. The rats were randomly divided into six groups and treated daily for 12 weeks as follows: Control group (Con, n = 8): rats received saline (1 mL/kg/d intravenously, i.v.); Control plus LC 50 group (Con+LC50, n = 8): rats received saline and LC (50 mg/kg/d i.v.); Control plus LC 200 group (Con+LC200, n = 8): rats received saline and LC (200 mg/kg/d i.v.); Diabetic control group (DN, n = 8): diabetic rats received saline; Diabetes plus LC 50 group (DN+L50, n = 8): diabetic rats received LC (50 mg/kg/d i.v.); Diabetes plus LC 200 group (DN+L200, n = 8): diabetic rats received LC (200 mg/kg/d i.v.).

Diabetes was induced by a single intraperitoneal injection of STZ (65 mg/kg). Induction of diabetes was defined as the point at which blood glucose concentration was ≥ 16.7 mmol/L (300 mg/dL). LC treatment began at 2 weeks after confirmation of diabetes and was administered at two dosages of 50 or 200 mg/kg/d for 12 weeks. At 0, 4, 8, and 12 weeks, the animals were placed in individual metabolic cages (Tecniplast Gazzada S. a r.l., Italy), urine and blood samples were collected, rats were euthanized under ketamine anesthesia, and kidney tissues were rapidly removed for mor-

phological and molecular studies. Each group included eight rats at each time point. Dosages of LC used in this study were chosen based on previous reports [11, 19].

Our experimental protocol was approved by the Animal Care Committee of the Catholic University of Korea (CUMC-2016-0101-01).

Biochemical examination

Body weight (BW) was recorded periodically for each rat. Fasting blood glucose (FBG) concentration, serum creatinine (Scr) concentration, and 24-h urinary protein excretion (UPE) were measured at 0, 4, 8, and 12 weeks (Coulter Electronics, Inc., CA, USA).

Histopathology

Kidney sections were stained with Periodic acid-Schiff (PAS) and Masson's trichrome. Evaluation of tubulointerstitial fibrosis (TIF) was performed as previously described [11, 20].

Immunohistochemistry

Sections were incubated for ED-1 (Serotec Inc., UK) and Wilms tumor protein (WT-1, Santa Cruz Biotechnology, CA). The number of WT-1- or ED-1-positive cells was evaluated by counting a minimum of 50 glomeruli/specimen in each section at ×400 magnification using a color image autoanalyzer (Polygon program, TDI Scope Eye Version 3.5 for Windows; Olympus, Japan).

Electron microscopy

For transmission electron microscopy, the kidneys were removed and fixed. Sections were washed in 0.1 Mphosphate buffer (0.1 MPB). Isolated glomeruli were fixed in 50 mM sodium cacodylate buffer (pH 7.4) that contained 2% glutaraldehyde in paraformaldehyde for 30 minutes at 32°C, post fixed in 1% osmium tetroxide for 2 hours at 4°C, and dehydrated by treatment with a graded series of ethanol (5 minutes each in 50, 60, 70, 80, 90, and 95% and twice in 100%). Isolated glomeruli were treated with propylene oxide and embedded in Epon 812 according to standard procedures. One micrometer semithin sections were stained
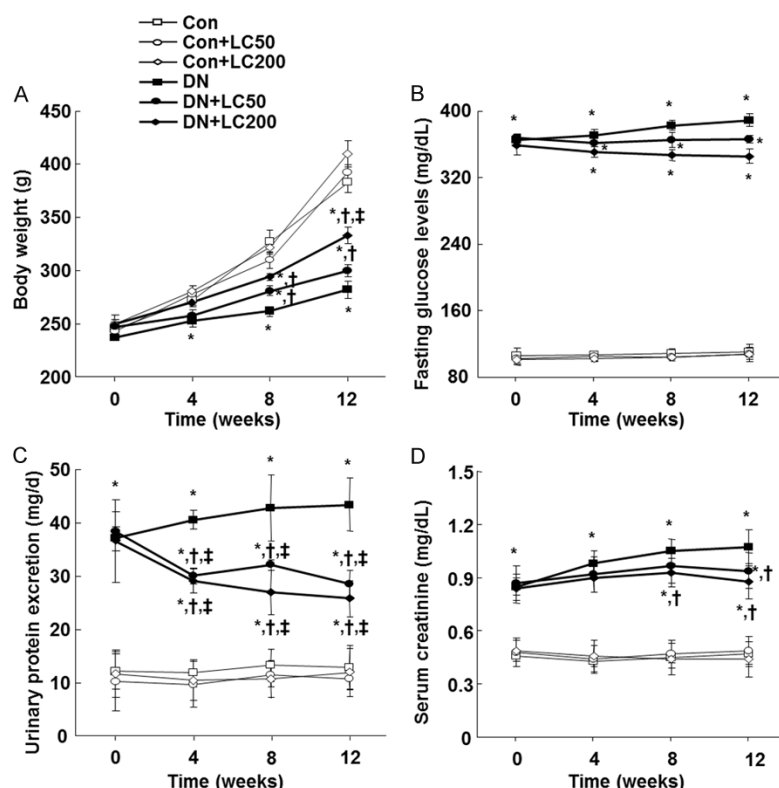
Figure 1. Basic parameters during the treatment period. A. Body weight; B. FBG; C. UPE; D. Scr. *$p<0.05$ vs. Con; †$p<0.05$ vs. DN; ‡$p<0.05$ vs. DN+LC50.

(Santa Cruz Biotechnology, CA, USA), active caspase-3 (Millipore, Billerica, Mass), and MnSOD (Abcam, Cambridge, MA) were detected with specific antibodies. Optical densities were obtained using the Con group as 100% reference and normalized with β-actin.

Enzyme linked immunosorbent assay

Urinary concentrations of DNA adduct 8-hydroxy-2'-deoxyguanosine (8-OHdG) were determined at 24 hours using a competitive enzyme-linked immunesorbent assay (Japan Institute for the Control of Aging, Shizuoka, Japan), according to manufacturer protocol. All samples were assayed in triplicate.
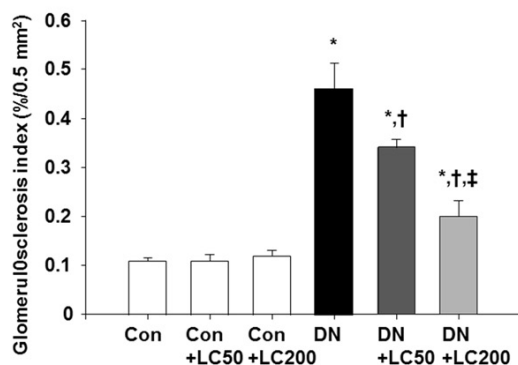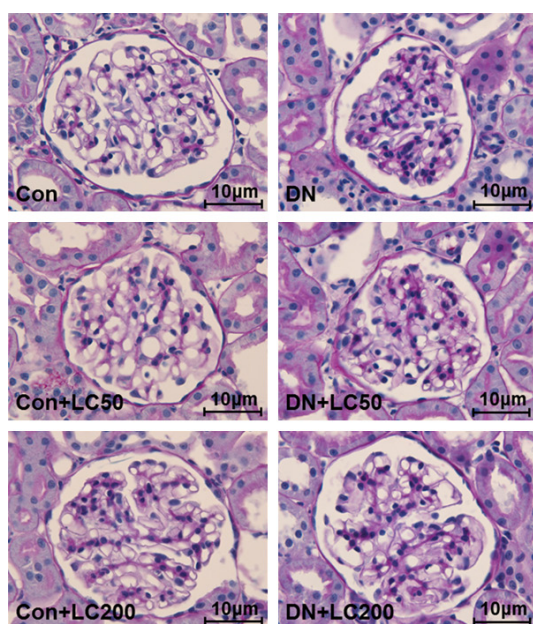
Statistical analysis

Data are expressed as mean ± SEM. For all variables, values were compared with those of the control group because there were no significant differences between control and control plus LC 50 and 200 groups at any time point. Multiple comparisons between groups were performed using one-way ANOVA and Bonferroni post hoc test using SPSS software (version 19.0; IBM, Armonk, NY). Statistical significance was accepted at $p<0.05$.

Results

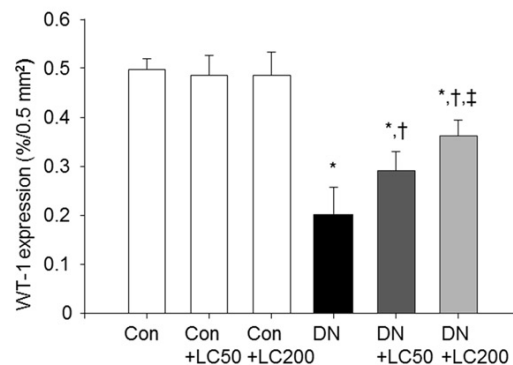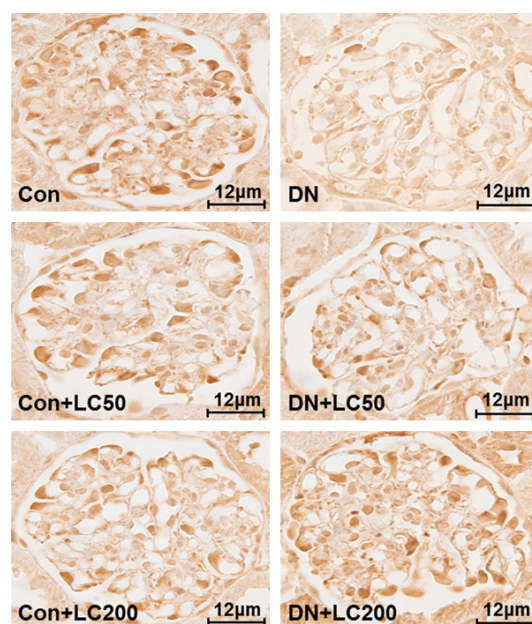*Effects of LC on basic parameters*

DN rats lost BW and exhibited an increase in FBG concentration. LC treatment at both dosages significantly prevented loss of BW (**Figure 1A**). FBG concentration did not differ significantly between experimental groups (**Figure 1B**). Renal dysfunction, as shown by increased UPE (**Figure 1C**) and Scr levels (**Figure 1D**) in DN rats, was significantly improved by administration of LC at both dosages compared with untreated diabetic rats.

with toluidine blue to visualize general cell morphology. Ultrathin sections were cut, stained with lead citrate and photographed with a transmission electron microscope, JEOL 1200EX (Tokyo, Japan). Two adjacent toluidine blue stained semi thin sections 3 um apart were observed in pairs at a magnification of ×10000, and nuclei present in the top section but not in the bottom section were counted and summed. Ten glomeruli in five rats from each group and 11 to 13 semithin sections from the mid glomerular area were examined.

Immunoblotting

Immunoblotting analyses were performed as previously described [11]. Monocyte chemotactic protein-1 (MCP-1, Santa Cruz Biotechnoogy, Inc., USA), toll-like receptor-2 (TLR-2, Santa Cruz Biotechnoogy, Inc., USA), transforming growth factor-beta1 (TGF-β1, R&D Systems, Minneapolis, MN, USA), TGF-β-inducible gene-h3 (βig-h3, Proteintech, Chicago, IL, USA), NF-κB (Santa Cruz Biotechnoogy, Inc., USA), I-κB (Santa Cruz Biotechnoogy, Inc., USA), Bcl-2

Figure 2. Representative photomicrographs of PAS staining and semiquantitative analyses. Original magnification (×400). *$p<0.05$ vs. Con; †$p<0.05$ vs. DN; ‡$p<0.05$ vs. DN+LC50.

Figure 3. Representative photomicrographs of immunohistochemistry for WT-1 and semiquantitative analyses. Original magnification (×400). *$p<0.05$ vs. Con; †$p<0.05$ vs. DN; ‡$p<0.05$ vs. DN+LC50.

Effects of LC on histopathology

Rats with diabetes-induced renal injury showed typical histological features of glomerulopathy, characterized by glomerular hypertrophy, mesangial expansion, thickening of the basement membrane, arteriolar hyalinosis, and nodular or sclerosis (**Figure 2**). As assessed by our semi-quantitative scoring system, GSI was higher in the DN group than the Con group (0.453 ± 0.059%/0.5 mm² *vs.* 0.142 ± 0.032%/0.5 mm², $p<0.01$). GSI was decreased markedly by LC administration in the lower dosage group (DN+LC50, 0.369 ± 0.043%/0.5 mm² *vs.* DN, $p<0.05$) and was decreased further in the higher dosage group (DN+LC200, 0.203 ± 0.031%/0.5 mm² *vs.* DN+LC50, $p<0.05$). To

detect podocyte injury, we used immunohistochemistry to examine WT-1 immunoreactivity. Glomerular expression of WT-1 was abundant in the Con group, as shown in **Figure 3**, whereas its immunoreactivity and number of WT-1-positive glomeruli were significantly lower in the DN group than in the Con group (0.208 ± 0.018%/0.5 mm² *vs.* DN, $p<0.05$). LC treatment preserved WT-1 expression and increased number of WT-1-positive cells. These effects were greater at the higher dose of LC (0.294 ± 0.053%/0.5 mm² *vs.* DN, 0.385 ± 0.023%/0.5 mm² *vs.* DN+LC50, $p<0.05$, respectively). Transmission electron microscopy showed that diabetic glomeruli exhibited severe damage such as glomerular basement membrane thickening and broadening of podocyte foot process
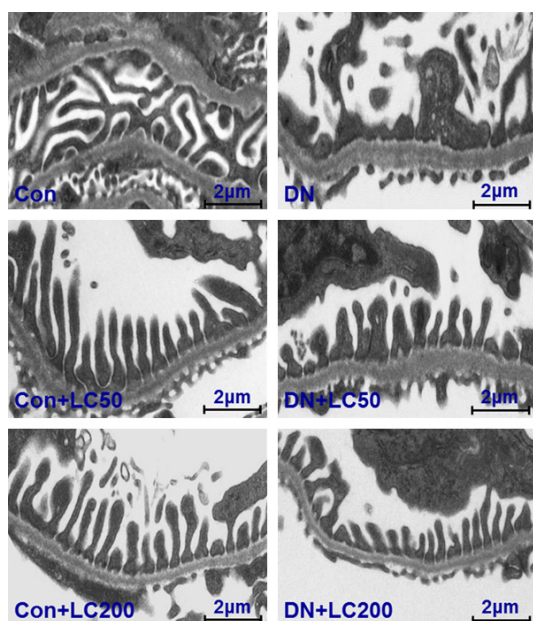
Figure 4. Representative photomicrographs of transmission electron microscopy for podocytes.

effacement, which is associated with a significant reduction in number of podocyte per glomerulus. By contrast, administration of LC preserved the ultrastructural appearance of podocytes (**Figure 4**).

Effects of LC on inflammation

ED-1-positive cells were rarely detected in glomeruli in the Con group (**Figure 5**, left panel), but the number increased significantly in glomeruli of the DN group (0.475 ± 0.016%/0.5 mm^2 *vs.* Con, $p<0.05$). Administration of LC decreased the number of ED-1-positive cells (0.343 ± 0.036%/0.5 mm^2 *vs.* DN, $p<0.05$). This decrease was more prominent at the higher dosage than lower dosage (0.206 ± 0.042%/0.5 mm^2 *vs.* DN+LC50, $p<0.05$). Immunoblotting showed markedly greater expression of MCP-1 and TLR-2 in the DN group than in the Con group (MCP-1: 168.4 ± 29.3% *vs.* 100.3 ± 2.1%; TLR-2: 221.4 ± 32.7% *vs.* 100.6 ± 2.4%, $p<0.01$, respectively). LC dose dependently decreased the expression of these two proteins (**Figure 6**).

Effects of LC on TGF-β1 and βig-h3 expression

Immunoblotting revealed that diabetic rats displayed a marked upregulation in expression of TGF-β1 (279.2 ± 16.8% *vs.* 100.4 ± 5.3%, $p<0.01$) and βig-h3 (240.2 ± 21.5% *vs.* 101.8 ±
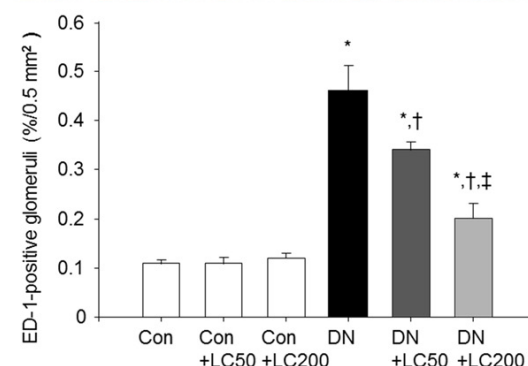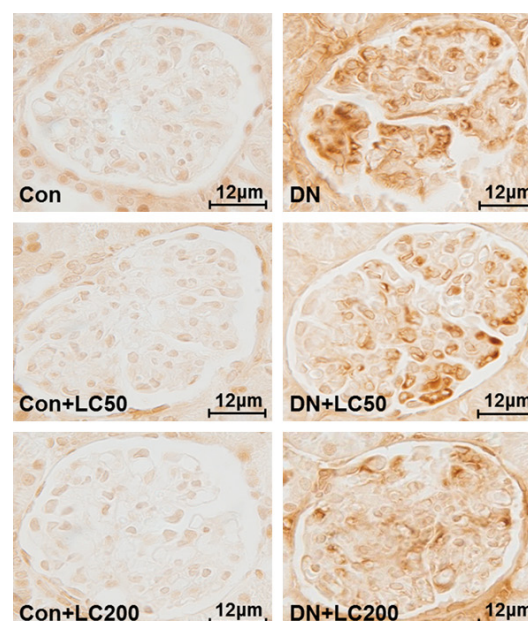
Figure 5. Representative photomicrographs of immunohistochemistry for ED-1 and semiquantitative analyses (×400). *$p<0.05$ vs. Con; †$p<0.05$ vs. DN; ‡$p<0.05$ vs. DN+LC50.

3.2%, $p<0.01$) compared with the Con group, whereas LC suppressed their expression in a dose-dependent manner (**Figure 7**).

Effects of LC on NF-κB

Induction of DN increased NF-κB protein expression (249.2 ± 12.9% *vs.* 108.3 ± 2.3%, $p<0.01$), accompanied by inhibition of I-κB protein expression (50.2 ± 9.7% *vs.* 100.8 ± 2.7%, $p<0.01$). However, this pattern was reversed by LC in a dose-dependent manner (**Figure 8**).

Effects of LC on apoptosis

Apoptotic cell death is attributed to the loss of podocyte in DN. We compared apoptosis-related gene expression between treatment groups.
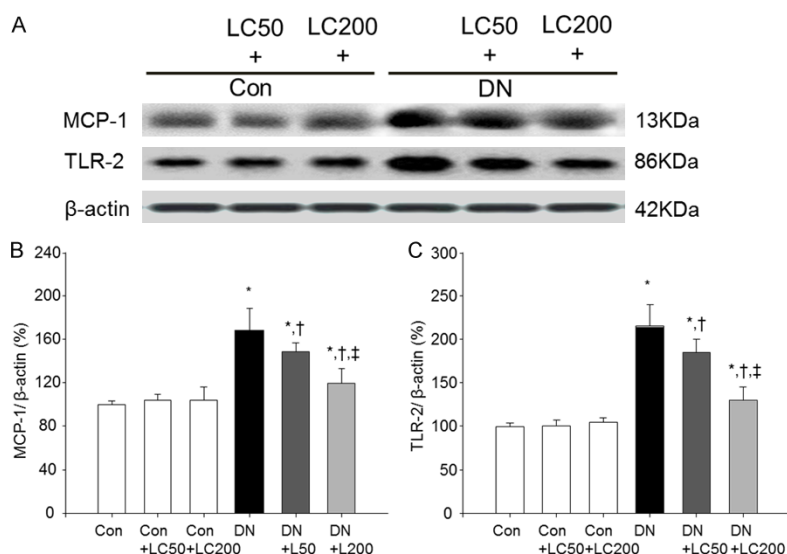
Figure 6. Immunoblotting (A) and desitometric analysis (B and C) for MCP-1 and TLR-2 in the treatment groups. MCP-1 and TLR-2 protein expression values are represented relative to the Con reference group, which is designated as 100% and are normalized to β-actin. *$p<0.05$ vs. Con; †$p<0.05$ vs. DN; ‡$p<0.05$ vs. DN+LC50.
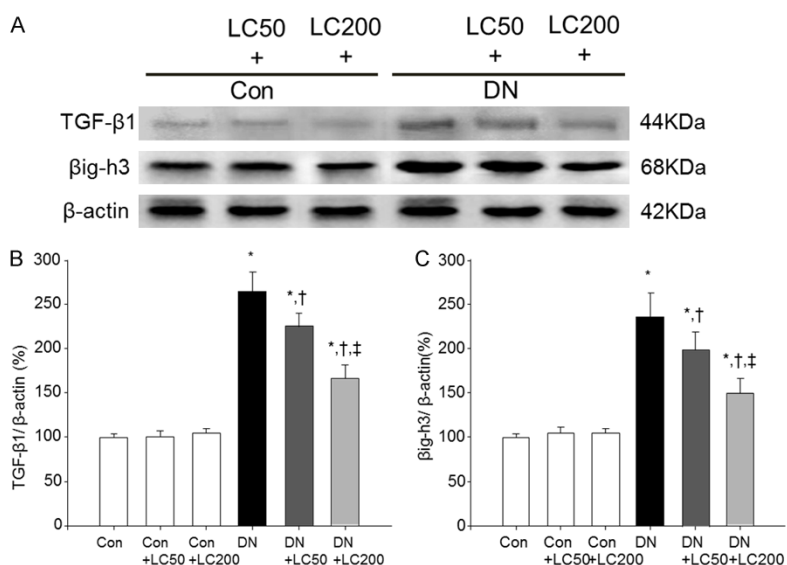
Figure 7. Immunoblotting (A) and desitometric analysis (B and C) for TGF-β1 and βig-h3 in the treatment groups. TGF-β1 and βig-h3 protein expression values are represented relative to the Con reference group, which is designated as 100% and are normalized to β-actin. *$p<0.05$ vs. Con; †$p<0.05$ vs. DN; ‡$p<0.05$ vs. DN+LC50.

Bcl-2 expression was downregulated (42.3 ± 3.1% vs. Con, $p<0.05$) in the DN group, but active caspase-3 was upregulated (172.6 ± 9.8% vs. Con, $p<0.05$). LC treatment at a dosage of 50 mg/kg/d significantly reversed these changes in expression of Bcl-2 and active cas-

pase-3 proteins (Bcl-2: 56.3 ± 3.1% vs. DN; caspase-3: 143.9 ± 2.4%, $p<0.05$, respectively). This effect was more pronounced at the higher dosage of 200 mg/kg/d (Bcl-2: 76.3 ± 2.5% vs. DN+LC50; caspase-3: 121.7 ± 3.8%, $p<0.05$, respectively) (**Figure 9**).

Effects of LC on oxidative stress

We examined markers of oxidative stress by measuring MnSOD expression and urinary 8-OHdG excretion in the treatment groups. As shown in **Figure 10**, MnSOD expression was suppressed in diabetic rats (56.4 ± 4.5% vs. 100.5 ± 2.7%, $p<0.01$) and was markedly upregulated by LC treatment in a dose-dependent manner (DN+LC50: 64.7 ± 3.8% vs. DN; DN+LC200: 79.6 ± 7.9% vs. DN+L50, $p<0.05$, respectively). Urinary 8-OHdG excretion was 83.8 ± 9.3 ng/day in the Con group and increased to 165.2 ± 37.2 ng/day after induction of diabetes. By contrast, starting at 4 weeks and continuing throughout the treatment period, 8-OHdG excretion was significantly lower in the DN+LC50 (4W: 228.3 ± 12.9 ng/day vs. 255.9 ± 8.9 ng/day, $p<0.05$) and DN+LC200 (4W: 205.7 ± 15.3 ng/day vs. 255.9 ± 8.9 ng/day, $p<0.05$).

Discussion

Our present study clearly demonstrates that, in this rat model of STZ-induced DN, LC administration attenuates progression of glomerular inflammation and sclerosis by downregulating expression of pro-inflammatory and pro-sclerotic cytokines. These molecular modifications
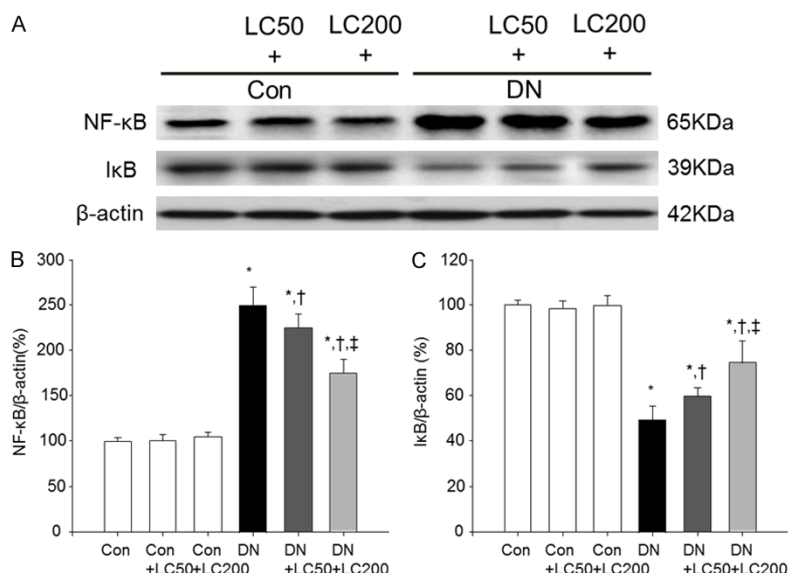
Figure 8. Immunoblotting (A) and desitometric analysis (B and C) for NF-κB and IκB in the treatment groups. NF-κB and IκB expression values are represented relative to the Con reference group, which is designated as 100% and are normalized to β-actin. $^*p<0.05$ vs. Con; $^\dagger p<0.05$ vs. DN; $^\ddagger p<0.05$ vs. DN+LC50.
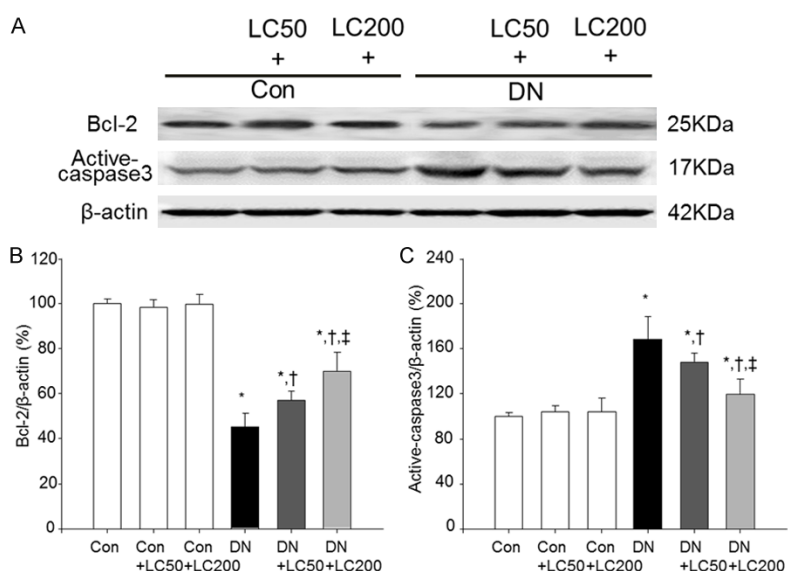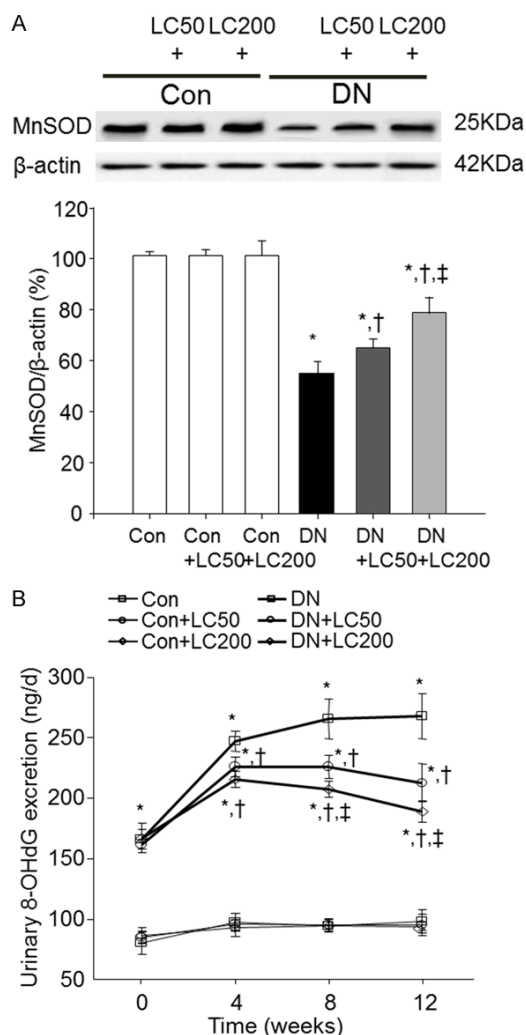

Figure 9. Immunoblotting (A) and desitometric analysis (B and C) for Bcl-2 and active caspase-3 in the treatment groups. Bcl-2 and active caspase-3 expression values are represented relative to the Con reference group, which is designated as 100% and are normalized to β-actin. $^*p<0.05$ vs. Con; $^\dagger p<0.05$ vs. DN; $^\ddagger p<0.05$ vs. DN+LC50.

were accompanied by recovery from both renal dysfunction and proteinuria. Of note, there was no significant difference in FBG level between groups. Our study demonstrates, for the first time, that LC may protect against DN.

LC inhibits pro-inflammatory cytokines (e.g. MCP-1, TNF-α, and IL-1) and macrophage infiltration and decreases TGF-β1 expression and extracellular matrix deposition in CsA- and contrast-induced renal injury [11, 13]. Therefore, LC may possess both anti-inflammatory and anti-fibrotic potential in kidneys. In this study, we found that LC significantly suppressed MCP-1, TLR-2, and TGF-β1 expression, and decreased the number of ED-1-positive cells in a dose-dependent manner. These changes were accompanied by marked improvement of renal dysfunction (UPE and Scr) and glomerulosclerosis (GSI). These findings are consistent with previous studies regarding anti-inflammatory and anti-fibrotic effects of LC in hemodialysis patients and rats with hypertension-associated renal fibrosis [15, 21]. These findings suggest that LC minimizes glomerular inflammation and sclerosis in STZ-induced DN through a mechanism involving decreased MCP-1, TLR-2, and TGF-β1 expression.

βig-h3 is a member of the extracellular matrix superfamily that interconnects different matrix components and resident cells to organize normal architecture of multiple organ systems including the kidney. However, its exact physiological and pathological functions are not well known. Accumulating evidence has demonstrated that upregulation of βig-h3 together with overexpression of TGF-β1 correlates with renal tubulointerstitial fibrosis and glomerulosclerosis in chronic CsA nephrop-

Figure 10. Immunoblotting for MnSOD (A) and urinary 8-OHdG concentrations (B) during the treatment period. MnSOD protein expression values are represented relative to the Con reference group, which is designated as 100% and are normalized to β-actin. $^*p<0.05$ vs. Con; $^†p<0.05$ vs. DN; $^‡p<0.05$ vs. DN+LC50.

athy and DN, implying a pathological role of βig-h3 in disease [21, 22]. As a result, βig-h3 expression is considered to provide an index of TGF-β1 bioactivity and degree of renal injury in human and rats [20, 23, 24]. In this study, we found that increased βig-h3 protein expression in diabetic rat kidneys was significantly decreased by LC administration in a dose-dependent manner. This effect was paralleled with downregulation of TGF-β1. Our findings suggest that renoprotective effects of LC in diabetic rat kidneys may be related in part to its action on βig-h3.

Podocyte injury is a hallmark of DN. Podocyte loss has been strongly associated with proteinuria, glomerulosclerosis, and decreased GFR in Pima Indians with type II diabetes [25, 26]. Several cell death pathways account for podocyte death and apoptosis may play a major role. Exposure of mouse podocytes to high glucose concentrations can cause apoptosis. For example, in Akita and db/db mice, about one-third of cells exposed to high glucose concentration underwent apoptotic cell death [27] and podocyte apoptosis coincided with onset of urinary albumin excretion and preceded significant loss of podocytes [28]. Here, we observed that LC upregulated Bcl-2 expression but downregulated active caspase-3, which favored cell survival. These changes were associated with increased WT-1 immunoreactivity and preservation of podocytes (transmission electron microscopy) in diabetic rat kidneys. These findings are consistent with earlier studies showing that LC attenuates hydrogen peroxide-mediated oxidative stress in a human tubule epithelial cell line (HK-2) through inhibition of cell apoptosis and protects renal tubular cells (NRK-52E) from gentamicin-induced apoptosis [29, 30]. Taken together, these findings suggest that LC exerts anti-apoptotic effects.

Mechanisms by which LC diminished glomerular inflammation and sclerosis in this study may be multifactorial, but two possibilities should be considered. First, oxidative stress caused by chronic hyperglycemia plays a critical role in development of DN [31]. This concept was confirmed by a study demonstrating that antioxidative agents inhibited MCP-1 expression in cultured podocytes and TGF-β1 production in glomerular mesangial cells. LC has been shown to improve endothelial dysfunction, diabetes-induced liver damage [32], and hyper-coagulation states [33] in diabetic rats by suppressing oxidative stress. Second, NF-κB is activated by numerous physiological and non-physiological stimuli. Activation of NF-κB, in turn, governs expression of many genes that play critical roles in the pathogenesis of various kidney diseases. It is well known that a high glucose concentration directly or indirectly induces MCP-1 or TGF-β1 upregulation [34, 35] in podocytes [36] and mesangial cells [37] via phosphorylation of the NF-κB signaling pathway. LC confers antiinflammatory and antiapoptotic effects by modulating NF-κB in contrast- and carboplatin-

induced renal injury [13, 38]. In this study, we found that LC inhibited urinary 8-OHdG excretion, increased production of antioxidant enzyme MnSOD and regulated NF-κB subunit expression (NF-κB and IκB) in DN rats. We propose that the anti-inflammatory and antisclerotic effects of LC observed in this study are associated with interference in both oxidative stress and NF-κB signaling pathways.

One aspect to consider when interpreting our results is the effect of LC on FBG level. It is generally accepted that LC augments glucose oxidation by increasing mitochondrial efflux of excess acyl groups and stimulating carnitine acetyltransferase, thereby causing feedback suppression of fatty acid oxidation by relieving inhibition of pyruvate dehydrogenase complex, the rate-limiting enzyme in mitochondrial glucose oxidation [39]. Therefore, LC may improve insulin sensitivity, glucose disposal rate, and glucose tolerance in patients with type 2 DM and severe insulin resistance [40, 41]. We and others have previously reported that LC treatment ameliorated the blood glucose level in STZ-induced diabetic rats that were fed a high-fat diet or with CsA-associated diabetes [11]. However, the opposite result was observed by Sleem et al., who reported that LC protected against endothelial dysfunction beyond its effect on blood glucose level in STZ-induced diabetic rats fed a normal diet. In this study, we found that LC did not affect FBG level at any time point in the treatment groups. Reasons for these discrepancies in the response of blood glucose level to LC remain unknown but they may reflect use of different conditions (e.g., diet) and models of DM.

In current clinical practice, LC supplements are used to treat carnitine deficiency caused by genetic disorders or ESRD requiring hemodialysis. However, LC exerts antioxidative activity and may play diverse roles. It has been reported that LC can improve vascular injury in patients on hemodialysis [42] as well as ventricular arrhythmia and angina symptoms in patients experiencing acute myocardial infarction [43]. In diabetes and metabolic diseases, LC improves insulin resistance, promotes glucose oxidation, and has beneficial effects on diabetic peripheral neuropathy and hepatic steatosis in nonalcoholic fatty liver disease and diabetes [44]. On the basis of our results and previously outlined reports, it is likely that add-ing LC to diabetes treatment as early as possible may provide extra benefits beyond attenuating DN. Future clinical trials are required to further investigate this issue.

In summary, our present study clearly shows that LC confers renoprotection through anti-inflammatory and anti-sclerotic effects in a rat model of DN. Inhibition of oxidative stress and NF-κB may be two underlying mechanisms of the beneficial effects of LC. Our study may provide a new avenue for administration of LC in the management of DN.

Disclosure of conflict of interest

None.

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