Effects of L-Carnitine Treatment on Kidney Mitochondria and Macrophages in Mice with Diabetic Nephropathy

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Keywords

Diabetic nephropathy · L-Carnitine · Mitochondria · Macrophage · Toll-like receptor 9 · Tumor necrosis factor- α

Abstract

Introduction: In diabetic nephropathy (DN), mitochondrial dysfunction and leakage of mitochondrial DNA (mtDNA) are caused by the downregulation of superoxide dismutase 2 (SOD2). mtDNA induces the activation of Toll-like receptor (TLR) 9, which is present in macrophages (M ϕ s), and triggers their activation. **Methods:** We orally administered L-carnitine, which exerts protective effects on the mitochondria, to obesity-induced DN (db/db) mice for 8 weeks. We then investigated the effects of L-carnitine on kidney mitochondrial reactive oxygen species (mtROS) production, circulating mtDNA content, and kidney CD11b^{high}/CD11b^{low} M ϕ functions. **Results:** In db/db mice, mtROS production increased in proximal tubular cells and kidney CD11b^{low} M ϕ s; both M ϕ types showed enhanced TLR9 expression. L-Carnitine treatment suppressed mtROS production in both proximal tubular cells and CD11b^{low} M ϕ s ($p < 0.01$), with improved SOD2 expression in the kidney ($p < 0.01$), decreased circulating mtDNA content, and reduced albuminuria. Moreover, it sup-

pressed M ϕ infiltration into kidneys and reduced TLR9 expression in M ϕ s ($p < 0.01$), thereby lowering tumor necrosis factor- α production in CD11b^{high} M ϕ s ($p < 0.05$) and ROS production by CD11b^{low} M ϕ s ($p < 0.01$). Collectively, these changes alleviated DN symptoms. **Conclusion:** The positive effects of L-carnitine on DN suggest its potential as a novel therapeutic agent against obesity-linked DN.

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Introduction

Diabetic nephropathy (DN) affects approximately 40% of patients with diabetes and is the major cause of chronic kidney disease worldwide [1, 2]. End-stage kidney disease may be the most recognizable result of DN; however, most patients actually die of cardiovascular disease and infection before kidney replacement therapy is performed [2]. In many cases, disease progression cannot be prevented by only controlling blood sugar or blood pressure to optimum levels. Therefore, to develop better therapeutics against DN, its molecular pathogenesis must be further elucidated.

Mitochondrial dysfunction is involved in DN pathogenesis [3, 4]. This dysfunction is related to the down-regulation of superoxide dismutase 2 (SOD2), which is localized in the mitochondria, and normally eliminates mitochondrial reactive oxygen species (mtROS) [5–8]. In an obesity mouse model fed with a high-fat diet, mitochondrial dysfunction enhanced mtROS production and damaged vascular endothelial cells, podocytes, and tubular cells via the inhibition of mitochondrial lipid β -oxidation and intracellular lipid accumulation [9]. These changes then cause proteinuria and interstitial inflammation.

In a septic mouse model, mitochondrial dysfunction increased circulating mitochondrial DNA (mtDNA) levels, which stimulated Toll-like receptor (TLR) 9 production in macrophages (M ϕ s) and exacerbated kidney injury through elevated inflammation [10]. Further, bacterial DNA with unmethylated CpG motifs has been shown to stimulate TLR9 action, which protects against bacterial infection through its involvement in tumor necrosis factor- α (TNF- α) production [11, 12]. In metabolic syndromes and obesity (underlying conditions that increase diabetes risk), TLR9 activates innate immunity and induces inflammation by recognizing cell-free DNA released into circulation from dead cells or adipocytes [13]. Furthermore, dysfunction-generated mtROS stimulate M ϕ s and cause NF- κ B-mediated inflammation [14].

Both M ϕ -driven TNF- α and ROS are involved in DN pathogenesis [15–18]. In the kidney, the M ϕ population is primarily composed of the CD11b^{low}F4/80^{high} and the CD11b^{high}F4/80^{low} subsets [19, 20]. In our earlier studies using metabolic-syndrome mouse models, we classified hepatic M ϕ s into two types: bone marrow-derived CD11b^{high} M ϕ s with high TNF- α production capacity and tissue-resident CD11b^{low} M ϕ s with ROS production capacity and phagocytic activity [21–25]. Enhancement of TNF- α and ROS production is associated with organ damage through chronic inflammation.

Currently, as a treatment for DN, angiotensin II receptor blocker and sodium glucose cotransporter 2 inhibitors have been used, in addition to optimizing blood glucose levels, to eliminate glomerular hyperfiltration. However, these agents do not have an adequate urinary protein reduction effect on advanced DN, which is associated with an increased urinary protein level. Furthermore, no treatment directly ameliorates the mitochondrial abnormalities of the kidney and the resulting inflammation underlying the pathology of DN.

L-Carnitine acts as a fatty-acid carrier in the mitochondria, exerts protective effects on the mitochondria [26–

28], and restores SOD2 expression [29–32]. The compound is widely used for treating carnitine deficiency and erythropoiesis-stimulating factor-resistant anemia in dialysis patients. In addition, L-carnitine has been suggested to be effective for diabetes, diabetic peripheral neuropathy, and diabetic podocyte injury [33–35]; however, its efficacy through mitochondrial protection in DN has not been investigated.

In this study, we administered L-carnitine to an obese mouse model of type 2 diabetes (db/db mice) and investigated whether the protective effect of L-carnitine on the mitochondria in proximal tubules and kidney M ϕ s would mitigate DN. Furthermore, we examined whether this protection influences the proportion and function of two M ϕ types in the kidneys. We, thus, aimed to investigate whether L-carnitine could alleviate DN by suppressing local kidney inflammation through kidney mitochondrial protection.

Materials and Methods

Animals

Male diabetic db/db mice (8–20 weeks old; C57BLKS/J *lar-lepr^{db}/+Lepr^{db}*) and their control nondiabetic littermates (misty; C57BLKS/J *lar-m+/m+*) were purchased from CLEA (Tokyo, Japan). All mice were housed under controlled conditions with a 12-h light/dark cycle. Food and water were provided ad libitum.

L-Carnitine Oral Administration

The average water intake of db/db mice was about 10 mL/day from 8 to 16 weeks of age. The daily dose of L-carnitine L-tartrate (hereafter, L-carnitine; FUJIFILM Wako Pure Chemical, Osaka, Japan) was 25 mg/individual [36]. Therefore, 2.5 g/L solution of L-carnitine was prepared and administered in drinking water from 8 weeks to 16 weeks of age (LC group). This treatment age and period were selected because urinary albumin (Alb) excretion is apparent at 8 weeks of age and increases linearly up to 16 weeks of age in db/db mice. Thus, the selected age and period were considered to appropriately reflect the exacerbation period of DN. The control (vehicle) group was administered regular water.

Measurement of Metabolic Variables

Urine samples were collected in metabolism cages, and urinary Alb levels were measured using the competitive enzyme-linked immunosorbent assay (ELISA) (Exocell, Philadelphia, PA, USA). Serum creatinine and plasma glucose levels were measured using an enzymatic method (SRL, Tokyo, Japan).

Preparation of Kidney Single-Cell Suspension

Mice were euthanized under deep anesthesia to obtain their left kidneys after saline perfusion. Kidneys were treated with collagenase for 40 min at 37°C, filtered through a stainless-steel mesh, and dissolved. Dissociated kidney cells were resuspended in an isotonic 30% Percoll solution (Sigma, St. Louis, MO, USA), layered on an isotonic 60% Percoll solution, and centrifuged at 430 g for 30 min.

The layer between the two Percoll solutions is rich in mononuclear cells (MNCs). This was collected, resuspended in red blood cell lysis solution, and filtered through a 40- μ m polypropylene cell strainer. These methods are a modification of established protocols for collecting liver MNCs [37].

Flow Cytometry

Kidney MNCs were incubated with Fc-blocker (2.4 G2; BD Biosciences, Franklin Lakes, NJ, USA) to prevent nonspecific binding. For the identification of M ϕ s, kidney MNCs were stained with allophycocyanin-labeled anti-CD45 (30-F11; eBioscience, San Diego, CA, USA), FITC- or phycoerythrin (PE)-labeled anti-F4/80 (BM8; eBioscience), and PE-Cyanine5-labeled anti-CD11b (M1/70; eBioscience) antibodies. To measure M ϕ -infiltration of kidneys, samples were further stained with PE-labeled anti-Ly6G antibody (clone: 1A8-Ly6g; eBioscience) to exclude neutrophils that highly express Ly6G. Samples were then stained with PE-labeled TLR9 (J15A7; BD Biosciences) antibodies to identify TLR9 expression in M ϕ s. Immediately before incubation with TLR9 antibodies, cell suspensions were subjected to cell membrane permeabilization using a BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Biosciences). For proximal tubular cell identification, the cell suspension was incubated at 4°C for 15 min along with nonspecific Fc-blocker (clone: 2.4 G2; BD Biosciences), allophycocyanin-labeled anti-CD45 antibody (clone: 30-F11; eBioscience), and *Lotus tetragonolobus* lectin (LTL; Vector Laboratories, Burlingame, CA, USA), a marker of proximal tubular cells [38]. Fluorescence-activated cell sorting identified a CD45-negative cell group as containing nonleukocyte cells. Among them, LTL-positive cells were identified as proximal tubular cells. A sample without LTL was also analyzed to eliminate cellular autofluorescence.

Fluorescent MitoSOX™ red mitochondrial superoxide indicator (Thermo Fisher Scientific, Waltham, MA, USA) was added to kidney cell suspensions and incubated at 37°C for 10 min. Mitochondria-derived ROS production in the cell population was evaluated as the ratio of MitoSOX fluorescence-positive cells to total cells. Cell-specific autofluorescence was excluded by comparing a sample and a control without MitoSOX. This allowed the identification of MitoSOX fluorescence-positive cells.

Samples were then analyzed using a NovoCyte Flow Cytometer (ACEA Biosciences, Inc., San Diego, CA, USA). Isotype control antibodies corresponding to each fluorescently labeled antibody were used as needed. The gating strategy is shown in online supplementary Figure 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000522013). The M ϕ -containing cell population was gated based on forward and side scatter, excluding neutrophils. However, considering that the gated cells contained kidney parenchymal cells (e.g., tubular cells), which must be excluded from M ϕ analysis, we gated lymphocytes and M ϕ -containing cells using the general leukocyte marker CD45. Next, we identified, among CD45⁺ cells, F4/80^{low} CD11b^{high} cells (CD11b^{high} M ϕ s) and F4/80^{high} CD11b^{low} cells (CD11b^{low} M ϕ s), along with lymphocytes. We also identified proximal tubular cells as LTL-positive among CD45⁺ cells. We then determined the proportion of mitochondrial-ROS-producing (mtROS⁺) cells in each cell population.

Evaluation of SOD2 Expression in Kidney Tissue

Immunohistochemistry with THE anti-SOD2 antibody was used to assess SOD2 expression in the kidneys, with emphasis on the tubules. Paraffin-embedded tissue sections were deparaffinized,

hydrophilized, and antigen-activated with a citrate buffer solution (pH 6) at 98°C for 1 h. After inhibition of nonspecific binding, tissue samples were incubated at 4°C for 24 h with a rabbit-derived, anti-SOD2, polyclonal primary antibody (ab13534; Abcam, Cambridge, UK), diluted 1,000-fold. Next, endogenous peroxidase in the tissue was inhibited, and samples were incubated with secondary antibody Histofine Simple Stain Mouse MAX PO (R) (Nichirei, Tokyo, Japan) at 20–24°C for 40 min and stained with diaminobenzidine. Nuclei were stained with Mayer's hematoxylin, and sections were mounted after dehydration and clearing. In each section, four fields of view were photographed randomly under $\times 20$ magnification. Using ImageJ-Fiji, SOD2-positive regions were extracted, and the positive area ratio was calculated [39].

Electron Microscopic Analysis

For electron microscopy, samples were fixed in 4% paraformaldehyde containing 0.25% glutaraldehyde. Ultrathin sections obtained from embedded Epon blocks were stained with uranyl acetate and lead citrate and examined using an electron microscope.

mtDNA Measurement

Total genomic DNA was extracted from the serum using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), and mtDNA content was quantified by TaqMan real-time PCR using a StepOnePlus Real-Time PCR System (Applied Biosystems) and mouse cytochrome b primers (Mm04225271; Thermo Fisher Scientific) [10]. The mouse ribosomal protein large P0 (*Rplp0*) primer (Mm00725448; Thermo Fisher Scientific) was used as a nuclear genome control to normalize the cytochrome b level [40–42]. Fold changes in gene expression were calculated using the $2^{-\Delta\Delta CT}$ method [43].

Evaluation of M ϕ Function

To assess TLR9-induced TNF- α production in M ϕ s, kidney cells rich in M ϕ s were re-stimulated in vitro with 20 ng/mL CpG-ODN (HC4033: TCCATGACGTTCTGATGCT; Hycult Biotechnology, Uden, The Netherlands) for 4 h; monensin (BD Biosciences) was used to block cytokine release. Cells were collected and stained to check for the surface expression of CD45, F4/80, and CD11b after incubation with Fc-blocker. Permeabilization was performed using a Fixation/Permeabilization Solution Kit (BD Biosciences), and cells were stained with fluorescein isothiocyanate (FITC)-labeled anti-TNF- α antibody (MP6-XT22; eBioscience).

To measure ROS production, kidney cells were incubated with the Fc OxyBURST™ Green Assay Reagent (Thermo Fisher Scientific) at 37°C for 30 min. The amount of ROS is expressed as a percentage of M ϕ s with positive fluorescence. Unstained cells were used to control for cell autofluorescence.

To assess phagocytic activity, kidney cells were incubated with FITC-labeled 0.75- μ m beads (Fluoresbrite YG Microspheres; Polysciences, Warrington, PA, USA) at 37°C for 30 min. Activity is expressed as a percentage of M ϕ -containing FITC beads.

Determination of Kidney TNF- α Levels

Proteins were extracted from kidney tissues using cell lysis buffer (radioimmunoprecipitation buffer; Wako) containing a 1% protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan) and subjected to an ELISA (TNF- α Mouse ELISA Kit; Thermo Fisher Scientific). Protein concentrations were determined using BCA

Fig. 1. Effects of L-carnitine on mtROS⁺ and mitochondria of proximal tubular cells. Percentage of mtROS⁺ cells in kidney CD11b^{high} Mφs (a), CD11b^{low} Mφs (b) (*n* = 8, 4, 10, and 12, respectively), and proximal tubular cells in the vehicle and the LC group of misty and db/db mice (c) (*n* = 4, 4, 6, and 8, respectively). Data are presented as means ± standard errors. Comparison between groups was performed using one-way analysis of variance with Tukey's HSD post hoc test. **p* < 0.05, ***p* < 0.01. Electron micrographs (×3,000) of proximal tubules in the db/db-vehicle (d) and the db/db-LC mice (e).

protein assay kits (Pierce; Thermo Fisher Scientific), and kidney TNF-α levels were evaluated per gram protein.

Histological Analysis

After removing kidney capsules, half of the right kidney was fixed in 4% paraformaldehyde solution for 1–2 days. Tissues were then embedded in paraffin for periodic acid-Schiff (PAS) and Masson trichrome staining, as well as podocyte quantification. Nuclei were counterstained with hematoxylin. Glomeruli PAS-stained sections were examined at ×400 by blinded observers. Glomerular mesangial expansion was scored semiquantitatively, with the percentage of mesangial matrix per glomerulus rated on a five-point scale as follows: grade 0, normal glomeruli; grade 1, mesangial expansion area up to 25%; grade 2, 25–50%; grade 3, 50–75%; and grade 4, >75% [16, 44, 45]. In Masson trichrome-stained sections,

four nonoverlapping low-power fields were randomly captured for analyzing the fibrotic area using ImageJ-Fiji [39].

Podocytes in paraffin-embedded sections were quantified after identification with Wilms tumor-I (WT-1) antibody (Santa Cruz Biotechnology, Dallas, TX, USA). Blinded observers counted WT-1⁺ cells in 10–20 glomeruli per section under ×40 magnification. Cell counts were averaged as described previously [46].

Statistical Analysis

Data are presented as means ± standard errors. Nonparametric Mann-Whitney U tests were performed to compare two groups, and one-way analysis of variance with Tukey's HSD post hoc test was performed to compare three or more groups. All statistical analyses were performed in JMP version 14 (SAS Institute Inc., Cary, NC, USA). Statistical significance was set at *p* < 0.05.

Fig. 2. Effect of L-carnitine on kidney SOD2 expression. **a** Kidney immunoperoxidase staining for SOD2 at $\times 20$ magnification in misty-vehicle, misty-LC, db/db-vehicle, and db/db-LC groups (upper panels). SOD2⁺ areas viewed in ImageJ-Fiji (lower panels). **b** Percentage of SOD2⁺ areas in the misty-vehicle, the misty-LC, the db/db-vehicle, and the db/db-LC mice ($n = 4, 4, 8$, and 8 , respectively). Data are presented as means $\pm$ standard errors. Comparison between groups was performed using one-way analysis of variance with Tukey's HSD post hoc test. ** $p < 0.01$.

Results

Effects of L-Carnitine Treatment on Mitochondrial ROS in Kidney Mφs and on Mitochondria in Proximal Tubular Cells

Misty-vehicle and db/db-vehicle mice did not exhibit differences in percentages of mtROS⁺ cells in kidney

CD11b^{high} Mφs (Fig. 1a). However, db/db-vehicle mice had significantly higher percentages of mtROS⁺ cells in kidney CD11b^{low} Mφs than in misty-vehicle mice ($p < 0.01$). L-Carnitine administration significantly lowered the mtROS⁺ cell percentage in CD11b^{low} Mφs among db/db mice ($p < 0.01$; Fig. 1b). Moreover, db/db-vehicle mice had a significantly higher percentage of mtROS⁺ cells in

Fig. 3. Effects of L-carnitine on serum mtDNA and physiological parameters of DN. **a** mtDNA content was measured as the mitochondrial (*CytB*) to nuclear (*Rplp0*) DNA ratio by qRT-PCR in the vehicle and the LC groups of misty and db/db mice ($n = 4, 4, 12$, and 6, respectively). Urinary Alb excretion (**b**), kidney weight (**c**), body weight (**e**), and blood sugar levels (**f**) were measured in the vehicle and the LC groups of misty and db/db mice ($n = 4, 8, 8$, and 12, respectively). Data are presented as means $\pm$ standard errors.

Comparison between groups was performed using one-way analysis of variance with Tukey's HSD post hoc test. $*p < 0.05$, $**p < 0.01$. **d** Number of leukocytes (CD45+ cells) infiltrating kidneys compared between the db/db-vehicle and the db/db-LC mice ($n = 8$ and 12, respectively). Comparisons were conducted using the non-parametric Mann-Whitney U test. $**p < 0.01$ versus the db/db-vehicle mice. *CytB*, cytochrome b; *Rplp0*, mouse ribosomal protein large P0.

Fig. 4. Effects of L-carnitine on kidney pathology in DN. **a** Kidney sections were PAS-stained for scoring glomeruli at $\times 160$ magnification. **b** PAS scores were compared between the db/db-vehicle and the db/db-LC mice ($n = 8$ per group). **c** Immunohistochemical staining to identify WT-1⁺ cells. **d** Number of WT-1⁺ cells/glomerulus compared between the db/db-vehicle and the db/db-LC mice ($n = 8$ per group). **e** Fibrotic areas were identified in Masson tri-

chrome-stained sections at $\times 20$ magnification (upper panels) and viewed using ImageJ-Fiji. **f** Percentage of fibrotic areas compared between the db/db-vehicle and the db/db-LC mice ($n = 8$ per group). Data are presented as means $\pm$ standard errors. Comparisons were conducted using the nonparametric Mann-Whitney U test. $**p < 0.01$ versus the db/db-vehicle mice.

proximal tubular cells than in misty-vehicle mice ($p < 0.01$). The addition of L-carnitine (db/db-LC) significantly lowered the percentage compared with that in the db/db-vehicle ($p < 0.01$; Fig. 1c). We also found that the db/db-LC mice had lesser mitochondrial swelling and cristae loss than the db/db-vehicle mice in proximal tubular cells (Fig. 1d, e).

Effect of L-Carnitine Treatment on Kidney SOD2 Expression

SOD2 expression in kidney tissue was significantly lower in db/db-vehicle mice than in misty-vehicle mice ($p < 0.01$) and significantly higher in db/db-LC mice than in db/db-vehicle mice ($p < 0.01$; Fig. 2a, b).

Effects of L-Carnitine Treatment on Serum mtDNA, Albuminuria, and Inflammation

Serum mtDNA was significantly higher in the db/db-vehicle mice than in the misty-vehicle mice ($p < 0.01$) and significantly lower in the db/db-LC mice than in the db/db-vehicle mice ($p < 0.05$; Fig. 3a). The db/db-LC mice excreted significantly less urinary Alb than the db/db-vehicle mice ($p < 0.01$; Fig. 3b), but the two groups did not differ in serum creatinine levels (data not shown). The db/db-LC mice also had a significantly lower kidney weight ($p < 0.01$) and number of kidney-infiltrating leukocytes (CD45⁺ cells; $p < 0.01$) than the db/db-vehicle mice (Fig. 3c, d). These results suggest that L-carnitine administration mitigated DN-induced inflammatory kidney enlargement. We observed no difference in body weight or blood sugar between vehicle and L-carnitine groups (Fig. 3e, f). The two groups of mice had similar food intake (data not shown).

Effects of L-Carnitine Treatment on Kidney Pathology in DN

The PAS-stained sections from db/db-LC mice showed reduced mesangial cell proliferation, alleviated glomerulosclerosis (Fig. 4a), and significantly lower glomerular PAS scores ($p < 0.01$; Fig. 4b). Furthermore, this group had significantly more glomerular WT-1⁺ cells (podocytes) ($p < 0.01$; Fig. 4c, d). Masson trichrome-stained sections revealed that db/db-LC mice exhibited significantly less kidney fibrosis ($p < 0.01$; Fig. 4e, f).

Effects of L-Carnitine Treatment on the Proportion and Function of Kidney Mφs in DN

The db/db-LC mice had fewer CD11b^{high} Mφs and more CD11b^{low} Mφs than the db/db-vehicle mice (Fig. 5a). Infiltration of both Mφ types to the kidney was

significantly lower in db/db-LC mice than in db/db-vehicle mice ($p < 0.05$; Fig. 5b, c). The former group also had a significantly lower CD11b^{high}/CD11b^{low} ratio ($p < 0.01$; Fig. 5d).

Effects of L-Carnitine Treatment on TLR9, TNF-α, ROS, and Phagocytosis in Kidney Mφs

TLR9 expression in CD11b^{high} Mφs was significantly higher in db/db-vehicle mice than in misty-vehicle mice ($p < 0.01$) and significantly lower in db/db-LC mice than in db/db-vehicle mice ($p < 0.01$), whereas it did not differ between misty-vehicle mice and misty-LC mice (Fig. 6a). TLR9 expression in CD11b^{low} Mφs was higher in db/db-vehicle mice than in misty-vehicle mice and significantly lower in db/db-LC mice than in db/db-vehicle mice ($p < 0.01$; Fig. 6b). TNF-α production in CD11b^{high} Mφs was significantly lower in db/db-LC mice than in db/db-vehicle mice (Fig. 6c). Additionally, homogenized kidney tissue from db/db-vehicle mice had a significantly higher TNF-α content than that from misty-vehicle mice ($p < 0.05$) and the tissue from db/db-LC mice had a significantly lower TNF-α content than that from db/db-vehicle mice ($p < 0.01$; Fig. 6d). The percentage of ROS-producing cells in CD11b^{low} Mφs was significantly higher in db/db-vehicle than in misty-vehicle ($p < 0.01$) and significantly lower in db/db-LC than in db/db-vehicle mice ($p < 0.01$; Fig. 6e). However, the percentage of phagocytic cells in CD11b^{low} Mφs was higher in db/db-LC than in db/db-vehicle mice ($p < 0.01$; Fig. 6f).

Discussion

In this study, we showed that (1) 8-week oral treatment with L-carnitine restored SOD2 and reduced mtROS levels in CD11b^{high} Mφs in proximal tubule cells and circulating mtDNA of db/db mice; (2) this drug downregulated the expression of TLR9 in Mφs, thereby reducing the TNF-α level and ROS release; and (3) these effects reduced the infiltration of CD11b^{high} Mφs into the kidney and restored the morphology of glomeruli, tubules, and the interstitium, while decreasing albuminuria in diabetic mice. We thus successfully demonstrated that L-carnitine administration alleviated DN in an obesity-induced type 2 diabetes mouse model.

Prior to L-carnitine treatment, we confirmed that mitochondrial dysfunction upregulated mtROS production in the kidneys of db/db mice, specifically in proximal tubular cells and CD11b^{low} Mφs. These results suggest that CD11b^{low} Mφs are more similar to tissue-specific cells,

Fig. 5. Effects of L-carnitine on proportion of kidney Mφs in DN. **a** Distributions of CD11b^{high} Mφs and CD11b^{low} Mφs were analyzed using fluorescence-activated cell sorting in the vehicle and the LC groups of misty and db/db mice ($n = 4, 4, 8$, and 12 , respectively). **b** Number of kidney CD11b^{high} Mφs in the db/db-vehicle and the db/db-LC mice ($n = 8$ and 12 , respectively). Comparisons were conducted using the nonparametric Mann-Whitney U test. * $p < 0.05$ versus the db/db-vehicle mice. **c** Number of kidney CD-

11b^{low} Mφs in the db/db-vehicle and the db/db-LC mice ($n = 8$ and 12 , respectively). Comparisons were conducted using the non-parametric Mann-Whitney U test. * $p < 0.05$ versus the db/db-vehicle mice. **d** Ratio of CD11b^{high}/CD11b^{low} Mφs ($n = 4, 4, 8$, and 12 , respectively). Data are presented as means $\pm$ standard errors. Comparison between groups was performed using one-way analysis of variance with Tukey's HSD post hoc test. ** $p < 0.01$.

Fig. 6. Effects of L-carnitine on the functions of kidney Mφs in DN. TLR9 expression in CD11b^{high} Mφs (a) and CD11b^{low} Mφs (b), as well as TLR9-induced TNF-α production, in CD11b^{high} Mφs compared among the vehicle and the LC groups of misty and db/db mice (c) (*n* = 4, 4, 8, and 8, respectively). d TNF-α content in kidney tissue homogenate compared among the vehicle and the LC groups of misty and db/db mice (*n* = 4, 4, 6, and 8, respectively). e Percentage of ROS-producing cells in CD11b^{low} Mφs among the vehicle

and the LC groups of misty and db/db mice (*n* = 4, 4, 7, and 8, respectively). f Percentage of phagocytic cells in CD11b^{low} Mφs compared among the vehicle and the LC groups of misty and db/db mice (*n* = 4, 4, 6, and 8, respectively). Data are presented as means ± standard errors. Comparison between groups was performed using one-way analysis of variance with Tukey's HSD post hoc test. **p* < 0.05, ***p* < 0.01.

Fig. 7. Schematic representation of the effects of L-carnitine on kidney Mφs. L-Carnitine administration restores SOD2 levels in the mitochondria of proximal tubular cells and CD11b^{low} Mφs, which alleviates mitochondrial damage and suppresses mtDNA

efflux, thereby inhibiting TLR9 levels in kidney Mφs and decreasing their TNF-α and ROS production. Through these mechanisms, L-carnitine contributes to morphological and functional improvements, along with decreased albuminuria, in *db/db* mice.

such as proximal tubular cells, than they are to CD11b^{high} Mφs. In support of this hypothesis, we previously showed that CD11b^{high} Mφs are derived from the bone marrow, whereas CD11b^{low} Mφs are tissue-resident in the liver [21–25].

Several studies have reported that SOD2 is downregulated in DN [6–8], and L-carnitine treatment restores its expression [29–32]. Here, we demonstrated that L-carnitine administration increased SOD2 expression in the kidney proximal tubules, coupled with decreasing mtROS production and improving mitochondrial morphology in proximal tubular cells. We therefore postulate that the protective effect of L-carnitine on the mitochondria is exerted through the restoration of mitochondrial SOD2 expression. The increased circulating mtDNA level in *db/db* mice was decreased by L-carnitine administration, suggesting that mitochondrial protection by L-carnitine reduced the amount of mtDNA flowing out from cells such as kidney tubular cells. L-Carnitine also reduced urinary Alb excretion and leukocyte infiltration in the kidney; alleviated inflammatory hepatic swelling, glomerular mesangial proliferation, and interstitial fibrosis; and restored podocyte levels.

Administration of L-carnitine decreased the populations of both kidney CD11b^{high} and CD11b^{low} Mφs. Par-

ticularly remarkable was the inhibition of CD11b^{high} Mφ infiltration into kidneys. L-Carnitine also downregulated TLR9 expression in both types of Mφs after DN-induced upregulation. Moreover, TNF-α production and levels decreased in CD11b^{high} Mφs and the kidney tissue homogenate, respectively.

As a further indication of its protective effect, L-carnitine reduced ROS production in CD11b^{low} Mφs. TNF-α and ROS production from CD11b^{high} and CD11b^{low} Mφs, respectively, exacerbates DN symptoms [15–18]. ROS production by Mφs is essential for maintaining homeostasis, but excess ROS causes inflammation and further tissue damage. Mφs produce ROS during phagocytosis and digestion of damaged tissue; thus, it is mandatory for maintaining homeostasis. We hypothesize that the mechanisms underlying the protective effects of L-carnitine and DN alleviation begin with restoring SOD2 expression, followed by inhibiting mtDNA efflux, suppressing TLR9, and inactivating kidney Mφs.

To further understand the underlying mechanisms of L-carnitine action, we focused on the relationship between glomeruli and tubules. In general, DN increases urinary Alb excretion through two routes: an abnormal slit membrane between foot processes of glomerular podocytes, causing Alb leakage, and impaired Alb reab-

sorption by tubules. Therefore, as depicted in Figure 7, L-carnitine may have corrected mitochondrial dysfunction in proximal tubule cells, thus suppressing tubule-derived mtDNA efflux. This then inhibited TLR9-mediated activation in kidney Mφs, stopping CD11b^{high} Mφ infiltration into glomeruli and Mφ production of TNF-α/ROS. Additionally, improved tubular function under L-carnitine treatment restores the Alb reabsorption capacity.

Elevated SOD2 levels, along with lower mtDNA and TLR9 levels, decrease the levels of CD11b^{low} Mφ-derived ROS [47]. L-Carnitine enhances the CD11b^{low} Mφ phagocytic ability by decreasing mtROS levels in CD11b^{low} Mφs and proximal tubular cells. This heightened phagocytosis may help prevent infection, repair damaged tissues, and alleviate nephropathy.

L-Carnitine binds to acyl-CoA that is converted from fatty acids, becomes a carrier for acyl-CoA (this binding complex is called acylcarnitine), and helps acyl-CoA to enter the inner mitochondrial membrane and undergo β-oxidation. In addition, it has been reported that administration of carnitine increases β-oxidation-related gene expression [48]. Therefore, carnitine presumably promotes β-oxidation in the proximal tubules of mice. Since excessive acyl-CoA is toxic to the mitochondria, carnitine, which potentiates the metabolism of β-oxidation, is thought to have a protective effect on the mitochondria [49, 50].

We did not clarify whether mitochondrial dysfunction occurs in diabetic Mφs or determine whether L-carnitine has a direct effect on Mφs. However, an increased population of mtROS + cells may reflect mitochondrial dysfunction (Fig. 1), which causes mtDNA leakage into the cytoplasm, upregulating the TLR9-MyD88 pathway [51]. Therefore, we speculate that systemic L-carnitine administration improved mitochondrial function in Mφs and suppressed mtDNA leakage, thereby also inhibiting TLR9 expression.

In large-scale clinical trials of patients with DN, glycemic control suppressed early DN onset and progression to overt DN [52, 53]. However, DN progression cannot be slowed once the disease becomes overt, even with current therapeutic measures, such as blood glucose treatment, blood pressure control, and renin-angiotensin-al-dosterone inhibitors [54]. Moreover, sodium glucose co-transporter 2 inhibitors are associated with improved cardiac metabolism and cardiac ATP production by reducing myocardial mtDNA damage and stimulating mitochondrial biosynthesis [55] and may be useful for kidney mitochondrial protection. Thus, for advanced-stage

DN, it is necessary to develop a therapy that targets the disease mechanism. Inhibition of Mφ infiltration into the kidney and regulation of its function through mitochondrial protection in the kidney are considered to be therapeutic methods that can be safely incorporated into existing therapies.

Our study had several limitations. First, although an increased level of circulating mtDNA was confirmed in DN mice, the mtDNA level in the kidney microcirculation was not investigated. However, in this study, since blood was collected in the inferior vena cava near the confluence of the kidney veins, it may reflect an increase in the mtDNA level in the kidney. Second, the direct relationship between changes in TLR9 expression and the amount of mtDNA was not investigated. Previous studies have shown that mtDNA can stimulate TLR9 and that increased cell-free DNA, which may include mtDNA, is associated with increased TLR9 expression [10, 13], but it is unclear whether mtDNA directly increases TLR9 expression in Mφs. It is considered that the decrease in TLR9 expression associated with L-carnitine administration reduced the chance of TLR9 stimulation by mtDNA and contributed to the alleviation of DN, but the decrease in TLR9 expression itself probably occurred independently of mtDNA.

In summary, L-carnitine treatment restored SOD2 and decreased mtDNA levels in proximal tubule cells and Mφs of db/db mice. Additionally, it downregulated TLR9 levels in Mφs, thereby dampening TNF-α levels and ROS release. These effects restored the morphology of the glomeruli and interstitium while decreasing albuminuria. As L-carnitine is safe for clinical use, we recommend it as a novel therapeutic agent against DN.

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Statement of Ethics

All animal experimental protocols were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All protocols were approved by the Animal Ethics Committee of the National Defense Medical College, Japan (approval number: 19004).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

S.I., M.N., and T.I. designed and performed the experiments under the supervision of S.S. and N.O. The manuscript was written by S.I., M.N., T.I., H.N., A.Y., T.I., M.K., S.S., H.K., and N.O. with the final version approved by all the authors.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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