Metabolism Clinical and Experimental 119 (2021) 154740

Contents lists available at ScienceDirect

Metabolism Clinical and Experimental

journal homepage: www.metabolismjournal.com

Apolipoprotein C3 aggravates diabetic nephropathy in type 1 diabetes by activating the renal TLR2/NF-κB pathway

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ARTICLE INFO

Article history:
Received 26 August 2020
Accepted 21 February 2021

Keywords:
Apolipoprotein C3
Type 1 diabetic nephropathy
Human mesangial cells
Triglyceride-rich lipoproteins
Toll-like receptor 2
Nuclear factor-κB

ABSTRACT

Objective: Apolipoprotein C3 (ApoC3) is a regulator of triglyceride metabolism and inflammation, and its plasma levels are positively correlated with the progression of diabetic nephropathy (DN) in patients. However, the role and underlying mechanism of ApoC3 in DN remain unclear.
Methods: Diabetes was induced in ApoC3 transgenic (Tg) and knockout (KO) mice by injection of streptozotocin. We studied the effect of ApoC3 on type 1 DN after 4 months of diabetes. Plasma glucose and lipid levels, renal function parameters and inflammation- and fibrogenesis-related gene and protein expression levels were studied. In vitro, human mesangial cells (HMCs) were incubated with high levels of glucose or/and triglyceride-rich lipoproteins (TRLs) with a high or low ApoC3 content isolated from Tg or wild-type (WT) mice, respectively, to explore the mechanisms of ApoC3 on development of DN.
Results: We found that compared to WT mice, Tg mice exhibited hypertriglyceridemia (HTG), aggravated early renal function injury and inflammation, enlarged glomerular and mesangial surface areas, renal lipid deposition and elevated fibrogenesis-related gene expression levels after 4 months of diabetes. ApoC3 overexpression activated the renal Toll-like receptor 2 (TLR2) and nuclear factor-κB (NF-κB) signaling pathways and increased the renal gene and protein expression levels of the downstream inflammatory factors TNF-α, VCAM-1 and MCP-1. Unfortunately, we did not find that ApoC3 deficiency had an obvious protective effect against DN. In vitro, we found that TRLs with a high ApoC3 content increased the gene and protein expression levels of inflammation- and fibrogenesis-related factors in HMCs compared to those following administration of the same concentration of TRLs with a low ApoC3 content. These effects of ApoC3 were inhibited by blockade of TLR2 or NF-κB.
Conclusions: These findings suggest that ApoC3 aggravates early-stage DN by activating the renal TLR2/NF-κB pathway which is partially independent of HTG.

1. Introduction

Diabetic nephropathy (DN) is a serious chronic microvascular complication in diabetes that leads to end-stage renal disease in as many as 30% of individuals with diabetes [1]. Patients with type 1 DN (T1DN) often exhibit elevated plasma triglyceride (TG) levels and renal inflammation [2,3]. These findings suggest that hypertriglyceridemia (HTG) and proinflammatory signaling pathways are important contributors to the progression of DN.

Apolipoprotein C3 (ApoC3), a regulator of TG metabolism and inflammation, is a small apolipoprotein (~8.8 kDa) secreted from the liver and small intestine that circulates on triglyceride-rich lipoproteins (TRLs), such as very-low-density lipoproteins (VLDLs) and chylomicrons, and on high-density lipoproteins [4]. ApoC3 increases TRLs and their remnants in the circulation primarily by blocking their catabolism

Abbreviations: ApoC3, apolipoprotein C3; DN, diabetic nephropathy; TRLs, triglyceride-rich lipoproteins; HTG, hypertriglyceridemia; TG, triglyceride; VLDLs, very low density lipoproteins; ASO, antisense oligonucleotide; CVDs, cardiovascular diseases; T1DN, type 1 DN; TLR2, Toll-like receptor 2; NF-κB, nuclear factor-κB; n-NF-κB, Nuclear NF-κB; Tg, transgenic; WT, wild-type; GLU, glucose; Dia, diabetic; ND, non-diabetic; TC, total cholesterol; FFA, free fatty acid; mApoC3, mouse ApoC3; hApoC3, human ApoC3; h, hours; UAE, urinary albumin excretion; UCR, urine creatinine; Ccr, creatinine clearance; KW/BW, kidney weight/body weight; PAS, periodic acid–Schiff; ORO, oil red O; PAI-1, plasminogen activator inhibitor-1; TGF-β, transforming growth factor-β; CTGF, connective tissue growth factor; KO, knockout; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TNF-α, tumor necrosis factor-α; VCAM-1, vascular cell adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; ICAM-1, intercellular adhesion molecule; IL-6, interleukin-6; IκBα, inhibitor of NF-κB α; HG, high glucose; HMCs, human mesangial cells; MA, mannitol; α-SMA, α-smooth muscle actin; Col IV, collagen IV.

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https://doi.org/10.1016/j.metabol.2021.154740
0026-0495/

and clearance and promoting VLDLs assembly and secretion [5,6]. Therefore, ApoC3 transgenic (Tg) mice exhibit HTG, whereas a null mutation in ApoC3 decreases TG levels in animals and humans [7–9].

Large-scale clinical studies have convincingly demonstrated that ApoC3 loss-of-function mutations decrease ApoC3 and TG levels and reduce the risk of cardiovascular diseases (CVDs) [10–12]. ApoC3 is considered a promising new drug target and novel interventional strategy for the treatment of CVD [13]. ApoC3 is also thought to be an independent cardiovascular risk factor for diabetes [14]. Elevated total ApoC3 concentrations are associated with a higher rate of diabetes [15]. Lowering ApoC3 levels with antisense oligonucleotides (ASOs) against ApoC3 mRNA delayed the onset of type 1 diabetes in rats and prevented diabetes-accelerated atherosclerosis in type 1 diabetic mice [16,17].

Volanesorsen, an ASO of ApoC3, decreased both ApoC3 production and TG concentrations in animals and healthy volunteers and was recently been approved in the EU for the treatment of adult patients with familial chylomicronemia syndrome [18]. Short-term treatment with volanesorsen reduced plasma ApoC3 and TG levels while improving integrative markers of diabetes in diabetic patients [19]. It is worth investigating the role of volanesorsen in the treatment of CVDs with or without diabetes in the future.

Diabetic macrovascular or microvascular complications have both a common pathogenesis and specific characteristics. Clinical studies have shown that plasma levels of ApoC3 are significantly increased in patients with T1DN and positively correlated with microalbuminuria levels [20–22]. These results suggest that ApoC3 is a risk factor for DN. However, the causal link between ApoC3 and T1DN is unknown. The effect of ApoC3 on T1DN has not been studied in animals. Therefore, in this study, we investigated the effect and possible mechanisms of ApoC3 on T1DN by using ApoC3 Tg and knockout (KO) mice.

In addition to regulating TG metabolism, ApoC3 modulates inflammation. As a ligand of Toll-like receptor 2 (TLR2), ApoC3 activates TLR2 and induces the activation of nuclear factor-κB (NF-κB) pathway-related inflammation and insulin resistance in skeletal muscle cells [23]. ApoC3 activates the NF-κB pathway in vascular endothelial cells, which causes monocytes and macrophages to adhere to endothelial cells, thereby activating downstream inflammatory factors, such as tumor necrosis factor-α (TNF-α), vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1) [24].

The persistent renal inflammation regulated by the TLR2/NF-κB pathway plays an important role in the pathogenesis of DN [3,25,26]. Increasing TLR2 expression was shown to induce MCP1 production downstream of the NF-κB pathway in mesangial cells, a major matrix-producing cell types [27]. Therefore, we investigated the effect of ApoC3 on TLR2/NF-κB pathway-related inflammation in ApoC3 Tg or KO mice with T1DN and in high glucose (HG)-stimulated human mesangial cells (HMCs). Our findings suggested that ApoC3 overexpression aggravated early-stage TIDN by activating the renal TLR2/NF-κB pathway with increased renal inflammation in mice. These effects of ApoC3 were inhibited by blockade of TLR2 or NF-κB in HG-stimulated HMCs, which was partially independent of TG levels.

2. Materials and methods

2.1. Animal experiments

ApoC3 Tg, ApoC3 KO and wild-type (WT) mice on a C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were maintained on a 12 h light/dark cycle and given ad libitum access to standard chow and water. All experiments involving mice were approved by the Institutional Animal Care Research Advisory Committee of Peking University Health Science Center and followed the Principles of Laboratory Animal Care (NIH Publication, 8th Edition, 2011).

Male ApoC3 Tg and littermate WT, as well as ApoC3 KO mice, at 8 weeks of age were divided into non-diabetic (ND) and diabetic (Dia) groups (n = 8–10): The diabetic (Tg-Dia, WT-Dia and KO-Dia) mice were given streptozotocin (60 mg/kg in 0.1 mol/L citrate buffer; pH 4.5) by intraperitoneal injection (i.p.) for 5–7 consecutive days to induce diabetes as described previously [28,29]. The ND (Tg-ND, WT-ND and KO-ND) mice were given citrate buffer i.p. After the induction of diabetes, only animals with plasma glucose (GLU) levels higher than 16.7 mmol/L were included in the study. Mice were studied for 4 months without insulin treatment.

2.2. Plasma glucose and lipid analyses

The mice were fasted for 4 h, blood samples were taken from the retroorbital plexus, and plasma was separated from the blood by centrifugation for 10 min at 4000 rpm. Plasma GLU, TG and total cholesterol (TC) levels were determined using enzymatic methods (Sigma, USA). Plasma free fatty acid (FFA) levels were measured using a nonesterified fatty acid assay kit (Wako, Japan). Plasma mouse ApoC3 (mApoC3) levels were measured using a mouse ApoC3 ELISA kit (CSB-EL0019333MO, Cusabio Biotech, China). Plasma human ApoC3 (hApoC3) levels in human ApoC3 Tg mice were measured using a human ApoC3 ELISA kit (ab154131, Abcam, USA).

2.3. Renal function parameters

After 1 day of adaptation in metabolic cages (Tecniplast, USA), 24 h urine samples were collected, and the food intake of the mice was measured. The urinary albumin concentration was measured with a mouse albumin ELISA kit (Bethyl Laboratories, USA). Urinary albumin excretion (UAE) was indicated by the excretion of albumin in 24 h urine. Urinary creatinine (UCR) was measured with a creatinine parameter assay kit (R&D Systems, USA). Plasma creatinine was measured by a mouse S—Cr ELISA kit (MM-44455 M2, Mmbio, Meimian, China). Creatinine clearance (Ccr) was calculated as the rate of urinary creatinine removal per minute divided by the plasma creatinine concentration (microliters per minute).

2.4. Histologic analysis of the kidney

The kidneys were cut transversally and fixed in 4% neutral formalin. Three-micrometer paraffin sections were stained with periodic acid–Schiff (PAS) and Masson trichrome stains. Seven-micrometer-thick frozen sections were stained with oil red O (ORO) stain. PAS staining without nuclear staining was used for morphometric analysis as previously described [28,30]. Assessment of the mesangial and glomerular surface areas was performed by pixel counts on a minimum of 50 randomly selected glomeruli per kidney section by ImageJ v.2.0 software (Image Processing and Analysis in Java; NIH; https://imagej.nih.gov/ij/). Kidney weight (KW) and body weight (BW) were also measured.

2.5. Isolation of TRLs

After 4 h of gavage with 200 μL of olive oil, plasma was separated from the heparin-treated whole blood of WT, ApoC3 Tg and KO mice by centrifugation. TRLs were isolated from plasma by ultracentrifugation for 3 h at 4 °C and 42,000 rpm (RC-5C Plus, Sorvall, USA). All procedures were carried out in a sterile manner. The TG content in TRLs was measured with a kit from Sigma as mentioned above.

2.6. Culture of HMCs

HMCs were cultured in modified RPMI 1640 medium (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA), 1% insulin transferase selenium (Sigma, USA) and 1% penicillin/streptomycin (Invitrogen, USA) as previously described [31]. HMCs were serum starved for 24 h before their medium was exchanged from the experimental medium. For the preliminary in vitro study, HMCs were stimulated with HG at

different concentrations (12.5, 25 mM) and/or TRLs (0.2, 0.4 mg/mL) for 6, 12 or 24 h, and mannitol (MA) was used as an isotonic control. We incubated HMCs with 25 mM glucose and 0.4 mg/mL TRLs for 12 h for the primary experiment. For the inhibition study, HMCs were treated with the NF-κB inhibitor BAY11–7082 (Cayman Chemical, USA) or siRNA targeting TLR2 (Santa Cruz, USA) (Supplementary Fig. S2) at different concentrations. For the formal experiment, HMCs were incubated with 2.5 μmol/L BAY11-7082 for 12 h with HG and TRLs. The cells were transfected with 80 nM siRNA targeting TLR2 for 48 h and then treated with HG and TRLs for 12 h. Total RNA or protein was extracted to detect related gene or protein expression levels, respectively.

2.7. RNA isolation and quantitative real-time PCR

Total RNA was extracted from tissues and cultured HMCs using TRIzol reagent (Invitrogen, USA), the concentration of RNA was measured using a Nanodrop 2000 (Thermo Fisher Scientific, USA) and first-strand cDNA was generated using the TransScript First-Strand cDNA Synthesis SuperMix kit (TransGen Biotech, China). Amplifications were performed in 35 cycles using an optional continuous fluorescence detection system (Stratagene Mx3000P, Agilent Technologies, USA) with EVAGreen fluorescence (ABM, Canada) as previously described [32]. Each cycle consisted of heating denaturation for 30 s at 94 °C, annealing for 30 s at 60 °C, and extension for 30 s at 72 °C. All samples were quantitated by using the comparative CT method for the relative quantitation of gene expression, with normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels as previously described [33]. Each sample was measured in triplicate. Sequences of the primers used in this study are shown in Supplementary Table S1. The results are represented by the ratio of the value to the control group value.

2.8. Western blot analysis

Total protein was extracted from mouse renal tissues or HMCs using RIPA solution, and nuclear proteins were extracted by using a high-salt buffer for measuring active NF-κB as previously described [33,34]. The protein concentration was determined by BCA protein assay (Thermo Fisher Scientific, USA). Then, denatured proteins were separated by sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). ECL chemiluminescence was used to detect the bands with an imaging system (molecular imager, ChemiDoc XRS, Bio-Rad, USA), and the densities of the bands were also determined semiquantitatively using the same system. Nuclear NF-κB (n-NF-κB) (p65) levels were normalized to the levels of histone H3, and the total protein levels were normalized to those of GAPDH. The results are represented by the ratio of the value to the control group value. Antibodies used in this study are summarized as follows: ApoC3 (ab55984, 1:1000, Abcam, USA), TLR2 (ab16894, 1:1000, Abcam, USA), VCAM-1 (ab134047, 1:1000, Abcam, USA), inhibitor of NF-κB α (IκBα) (4814T, 1:1000, Cell Signaling Technology, USA), P-IκBα (2859T, 1:1000, Cell Signaling Technology, USA), TNF-α (3707S, 1:1000, Cell Signaling Technology, USA), NF-κB (sc-8008, 1:500, Santa Cruz, USA), phospho-NF-κB (p65) (3033P, 1:1000, Cell Signaling Technology, USA), MCP-1 (sc-52701, 1:500, Santa Cruz, USA), histone H3 (BE3015, 1:1000, Easybio, China), GAPDH (ABS16, 1:5000, Millipore, USA), and blots were then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (ZSGB-BIO, China).

2.9. Statistical analysis

All data are presented as the means ± SEMs. Statistical comparisons were performed using 2-way ANOVA followed by Tukey's test or the Mann-Whitney *U* test for nonparametric data, and Student's *t*-test was used for the in vitro study. GraphPad Prism 7.0 software was used for statistical analyses. A value of $P < 0.05$ indicated a statistically significant difference.

3. Results

3.1. Effects of ApoC3 overexpression on body weight, food intake and main plasma biochemical parameters in ApoC3 Tg diabetic mice

To study the contribution of ApoC3 to DN, diabetes was induced in ApoC3 Tg mice. Decreased body weight and increased daily food intake were found in both WT-Dia and Tg-Dia mice compared with ND mice after diabetes (Fig. 1A–B). Plasma GLU levels increased to ~25 mmol/L in both Tg and WT mice after diabetes induction (Fig. 1C). Extremely high plasma TG levels and increased plasma TC and FFA levels were detected in Tg mice compared to WT mice in both the ND and Dia groups (Fig. 1D–F). Moreover, we found that plasma TG levels were elevated in WT-Dia mice compared with WT-ND mice after 4 months of diabetes (Fig. 1D). However, plasma TG, TC and FFA levels were not further increased in Tg-Dia mice at 2 and 4 months after the induction of diabetes (Fig. 1D–F). Plasma endogenous mApoC3 levels were 42.3 ± 3.9 μg/mL and 31.8 ± 3.4 μg/mL in WT-ND and Tg-ND mice, respectively, and were increased approximately 2-fold after diabetes (Fig. 1G). Plasma hApoC3 levels were 811 ± 20 μg/mL in Tg-ND mice and were not further increased after diabetes induction. Compared to that in WT mice, the total plasma ApoC3 levels were increased 19- and 10-fold in ApoC3 Tg mice before and after diabetes induction, respectively (Fig. 1G). The results of plasma ApoC3 levels were consistent with the change of plasma TG levels (Fig. 1D, G).

3.2. ApoC3 overexpression aggravated renal function injury in ApoC3 Tg diabetic mice

The 24 h urinary volume increased progressively with the development of diabetes in both WT and Tg mice (Fig. 2A). Diabetes had increased the 24 h UAE, 24 h UCR and Ccr levels in both WT and Tg mice at 2 months after diabetes, and these indicators in Tg-Dia mice were further increased at 4 months of diabetes (Fig. 2B, C and E). Moreover, at 4 months after diabetes, Tg-Dia mice had significantly elevated 24 h UAE, 24 h UCR and Ccr levels compared to those in WT-Dia mice (Fig. 2B, C and E). The plasma creatinine levels tended to be increased in both the WT and Tg groups after diabetes, but these differences were not statistically significant (Fig. 2D).

3.3. Effects of ApoC3 overexpression on renal pathologic abnormalities in ApoC3 Tg diabetic mice

At 4 months after diabetes, the KW/BW ratio was markedly higher in Dia mice than in ND mice (Fig. 3A). In the diabetic group, the KW/BW ratio increased more significantly in Tg-Dia mice than in WT-Dia mice (Fig. 3A). Quantitative analysis of PAS staining revealed that diabetes increased the glomerular and mesangial surface areas, and the increases in these areas were more pronounced in Tg-Dia mice than in WT-Dia mice at 4 months after diabetes (Fig. 3B–D). Glomerular lipid deposits were observed in Tg-Dia mice by ORO staining (Fig. 3B). No significant renal fibrosis was observed in Tg or WT mice with or without diabetes by Masson trichrome staining (Fig. 3B). These results indicated that ApoC3 aggravated early-stage T1DN after 4 months of diabetes.

3.4. Effects of ApoC3 deficiency on body weight, food intake, plasma glucose and lipid levels, renal function and histological changes in ApoC3 KO diabetic mice

To further observe the effects of ApoC3 on the progression of DN, we induced diabetes in ApoC3 KO mice. Reduced plasma TG levels were detected in KO mice compared to WT mice in both the ND and Dia groups (Table 1). Diabetes reduced body weight, increased daily food intake and aggravated renal function injury and pathologic abnormalities in both WT and KO mice (Table 1). Unfortunately, after 4 months of diabetes, we did not find that ApoC3 deficiency significantly improved

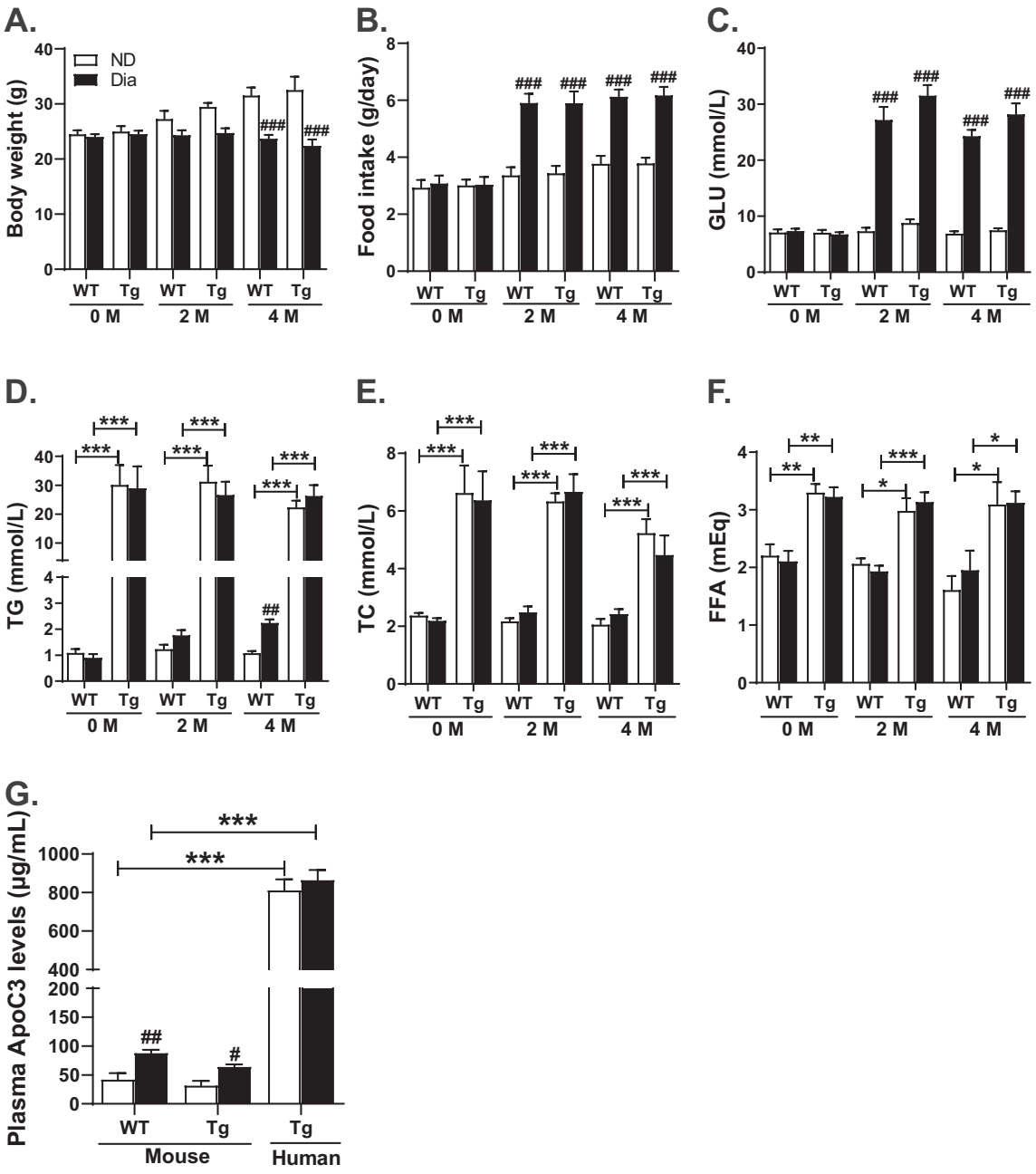

Fig. 1. Body weight, food intake, plasma glucose and lipid levels in WT and Tg mice under diabetic conditions. (A) Body weight, (B) food intake, (C) plasma glucose (GLU), (D) triglyceride (TG), (E) total cholesterol (TC) and (F) free fatty acid (FFA) levels at 0, 2 and 4 months after diabetes. (G) Plasma mouse and human ApoC3 levels at 4 months after diabetes. n = 8–10 mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. WT mice, effect of genotype. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. ND mice, effect of diabetes. WT, wild-type mice; Tg, ApoC3 transgenic mice; ND, non-diabetes; Dia, diabetes; M, months.

injured renal function or renal pathologic abnormalities, except the 24 h UCR level in the KO-Dia mice was lower than that in WT-Dia mice (Table 1). These results indicated that ApoC3 deficiency had no obvious protective effect on T1DN in mice.

3.5. ApoC3 overexpression activated the TLR2/NF-κB pathway and aggravated renal inflammation and fibrogenesis-related gene expression in ApoC3 Tg diabetic mice

As a ligand of TLR2, ApoC3 activates NF-κB, and HG can induce the increased expression of TLR2 [3,25]. Diabetes increased the renal gene and protein expression of TLR2 in both the WT and Tg groups compared to that in the ND group. Under diabetic conditions, the gene and protein expression levels of TLR2 were further increased in Tg-Dia mice compared with WT-Dia mice (Fig. 4A–C). NF-κB is an important regulator of inflammation. Diabetes increased expression levels of the renal NF-κB gene and n-NF-κB protein and the ratio of P-IκBα to total IκBα (P-IκBα/IκBα) in both the WT and Tg groups compared to the ND group. Under diabetic conditions, ApoC3 overexpression further increased the expression levels of the renal NF-κB gene and n-NF-κB protein and the P-IκBα/IκBα ratio (Fig. 4A–C).

We then studied the inflammatory factors downstream of the TLR2/NF-κB pathway. Diabetes elevated the renal gene and protein expression levels of TNF-α, VCAM-1 and MCP-1 in the WT and Tg groups compared with the ND group (Fig. 4A–C). Under diabetic conditions, the above parameters were further increased in Tg-Dia mice compared with WT-Dia mice (Fig. 4A–C). Elevated renal gene expression levels of intercellular adhesion molecule (ICAM-1), interleukin-6 (IL-6) and

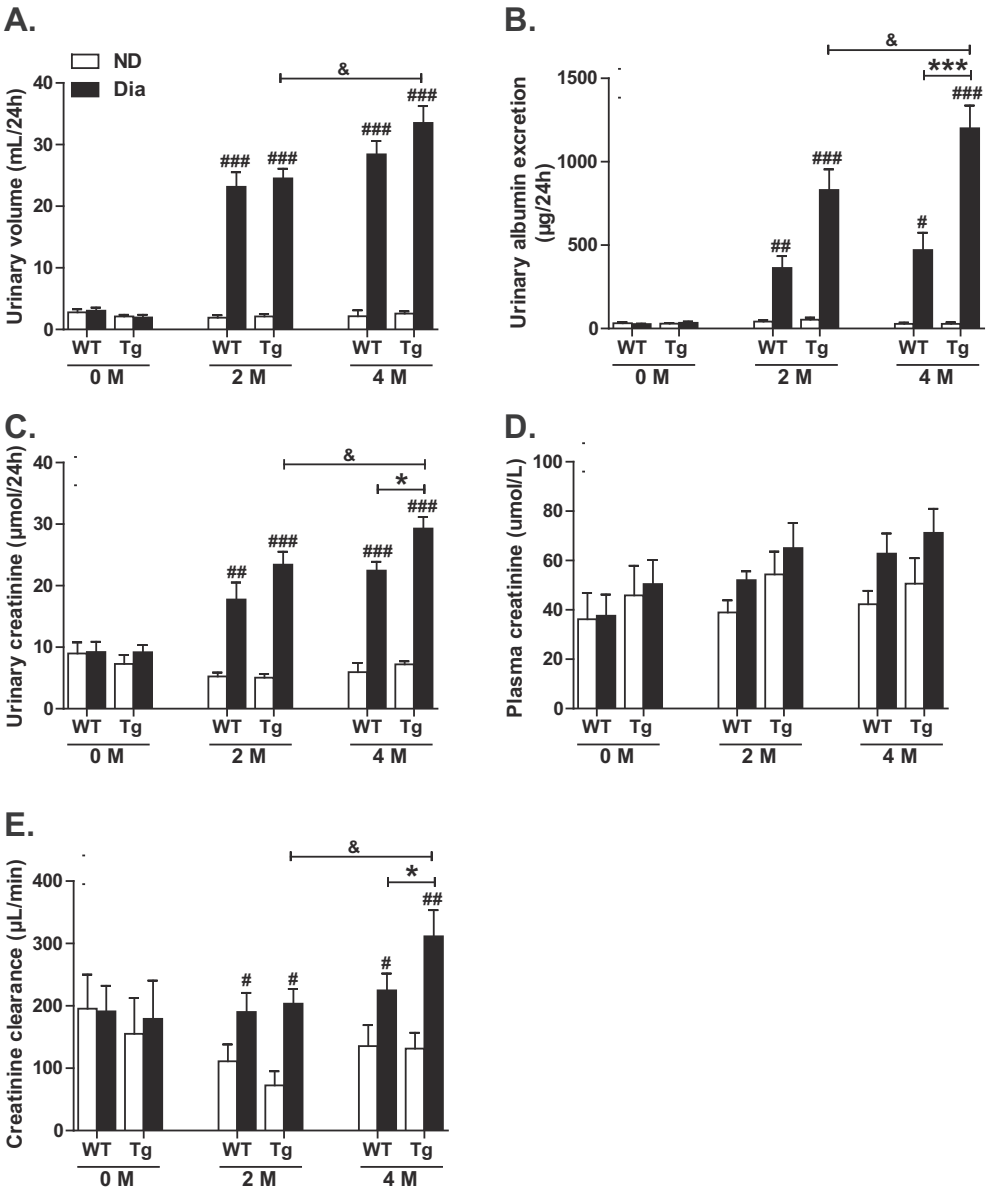

Fig. 2. Urine parameters and plasma creatinine levels in WT and Tg mice under diabetic conditions. (A) 24 h urinary volume, (B) 24 h urinary albumin excretion, (C) 24 h urinary creatinine, (D) plasma creatinine and (E) creatinine clearance at 0, 2 and 4 months after diabetes. n = 8–10 mice per group. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. WT mice, effect of genotype. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. ND mice, effect of diabetes. $^{\&}P < 0.05$, vs. 2 M, effect of duration. WT, wild-type mice; Tg, ApoC3 transgenic mice; ND, non-diabetes; Dia, diabetes; M, months.

IL-1β were detected in Tg-Dia mice but not in WT-Dia mice compared with ND mice (Fig. 4A).

Furthermore, diabetes significantly increased expression levels of the fibrogenesis-related genes transforming growth factor-β (TGF-β), plasminogen activator inhibitor-1 (PAI-1), connective tissue growth factor (CTGF) and fibronectin compared to their expression in the ND group. Under diabetic conditions, ApoC3 overexpression further increased TGF-β, CTGF and Fibronectin expression levels (Fig. 4D). Taken together, these results suggest that ApoC3 overexpression activated the renal TLR2/NF-κB pathway and inflammation, ultimately aggravating T1DN development in diabetic mice.

3.6. ApoC3 overexpression aggravated TRLs-induced inflammation by activating the TLR2/NF-κB pathway in HMCs with HG

To further explore the underlying mechanism of ApoC3 on DN development, we exposed HMCs to the same concentration of TRLs with a high or low ApoC3 content isolated from the plasma of Tg or WT mice, respectively, under HG conditions. The ApoC3 protein levels in ApoC3 Tg mouse-derived TRLs (TRLs-C3) were approximately 4-fold higher than those in WT mouse-derived TRLs (TRLs-WT) (Fig. 5A). Considering these preliminary results, we used 0.2 or 0.4 mg/mL TRLs to stimulate HMCs with 25 mM HG for 12 h in the following experiments (Supplementary Fig. S1). We found that 0.4 mg/mL TRLs-WT and TRLs-C3 increased NF-κB, TNF-α and VCAM-1 gene expression levels at both normal glucose and HG conditions, so we chose 0.4 mg/mL as the concentration of TRLs used for the final experiments (Fig. 5B–C).

Both HG and TRLs (TRLs-WT and TRLs-C3) clearly increased the protein levels of TLR2 and n-NF-κB (P65), P-IκBα/IκBα ratio, and protein levels of the downstream proinflammatory cytokines TNF-α, VCAM-1 and MCP-1 in HMCs (Fig. 5D–G). In the groups stimulated with TRLs alone, TRLs-C3 increased these protein levels to a greater degree than TRLs-WT (Fig. 5D–G). Under HG conditions, TRLs further increased the protein levels of the above factors; more importantly, TRLs-C3 elevated these protein levels to a greater degree than TRLs-WT (Fig. 5D–G). These results indicated that TRLs and HG increased inflammatory

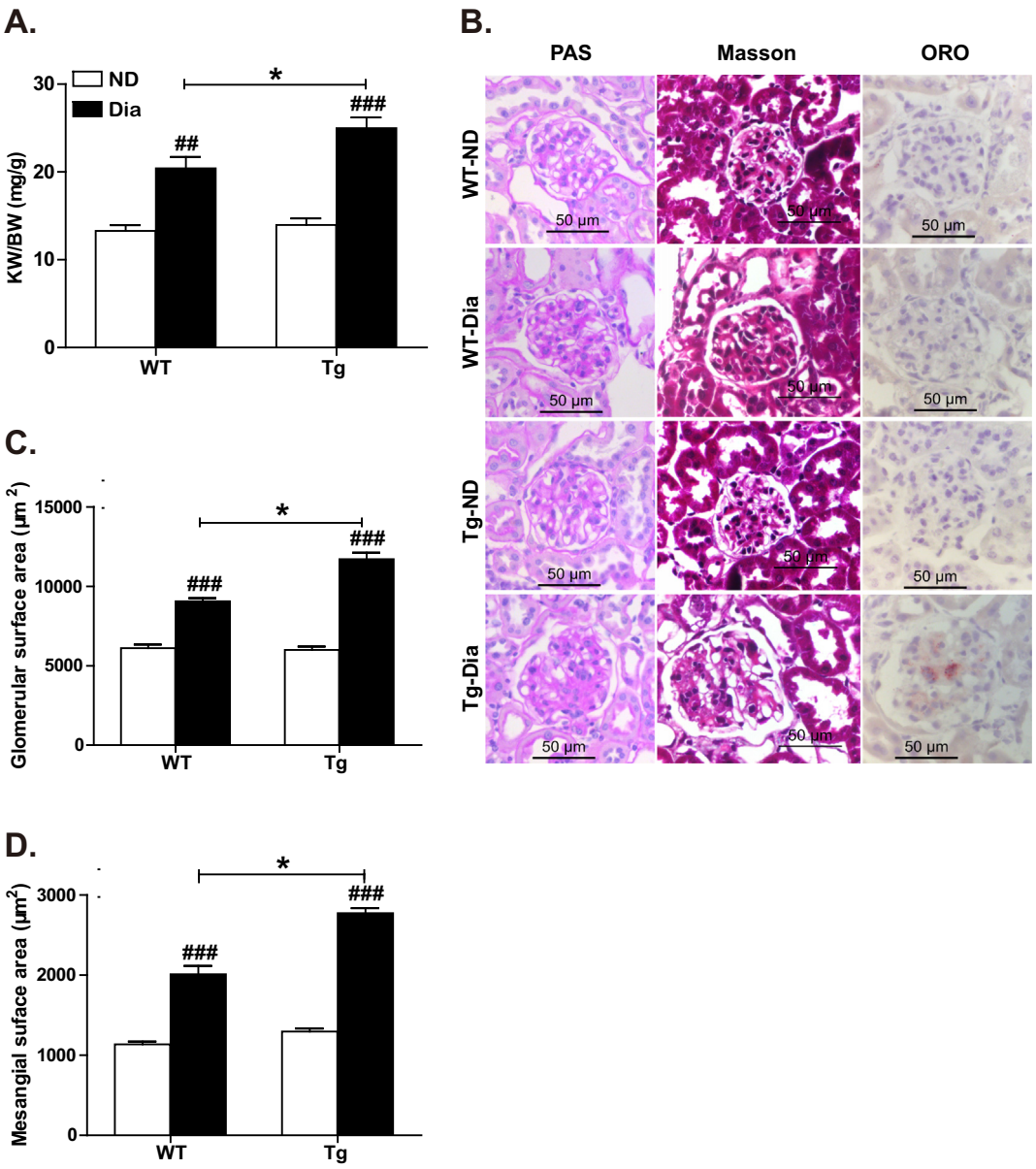

Fig. 3. The KW/BW ratio, renal morphology and quantitative analysis of glomerular and mesangial surface areas in WT and Tg mice under diabetic conditions. (A) KW/BW, (B) renal periodic acid–Schiff (PAS) staining, Masson trichrome staining and oil red O (ORO) staining (original magnification, ×400). Quantification of glomerular (C) and mesangial (D) surface areas. All data were collected at 4 months after diabetes. n = 8–10 mice per group. $^{*}p < 0.05$ vs. WT mice, effect of genotype. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. ND mice, effect of diabetes. WT, wild-type mice; Tg, ApoC3 transgenic mice; ND, non-diabetes; Dia, diabetes; KW/BW, kidney weight/body weight.

responses in HMCs and that TRLs with a high ApoC3 content had a greater effect than TRLs with a low ApoC3 content at the same concentration. These findings suggest that ApoC3 aggravated inflammation in HMCs independent of TG levels.

Based on the preliminary study, 2.5 μmol/L BAY11-7082 (an NF-κB inhibitor) was selected to stimulate HMCs treated with HG and TRLs-C3 (Supplementary Fig. S2A–B). BAY11-7082 decreased HG+TRL-C3-induced increases in the protein and/or mRNA expression levels of TLR2, n-NF-κB (P65), P-IκBα/IκBα and downstream proinflammatory cytokines TNF-α, VCAM-1, MCP-1, ICAM-1 and IL-6 (Fig. 5D, E and H).

Based on the preliminary experiment, HMCs transfected with 80 nM TLR2 siRNA were treated with HG and TRLs-C3 (Supplementary Fig. S2C). TLR2 siRNA decreased HG+TRL-C3-induced increases in the protein and/or mRNA expression levels of TLR2, n-NF-κB (P65), NF-κB, P-IκBα/IκBα and downstream proinflammatory cytokines TNF-α, VCAM-1 and MCP-1 and mRNA expression levels of ICAM-1 and IL-6 (Fig. 5F, G and I).

Overall, the above results suggest that ApoC3 overexpression aggravated TRLs-induced inflammation by activating the TLR2/NF-κB pathway in HMCs under HG conditions.

3.7. ApoC3 overexpression aggravated TRLs-induced increases in fibrogenesis-related gene expression by activating the TLR2/NF-κB pathway in HMCs under HG

TRLs increased expression levels of the fibrogenesis-related genes α-smooth muscle actin (α-SMA), collagen IV (Col IV), fibronectin, TGF-β and CTGF in HMCs, and TRLs-C3 increased these parameters to a greater degree than TRLs-WT (Fig. 6A). Under HG conditions, both TRLs-WT and TRLs-C3 further elevated the expression levels of these genes in HMCs,

Table 1
Body weight, food intake, plasma measurements and renal functions in WT and KO mice after 4 months of diabetes.

	WT-ND	WT-Dia	KO-ND	KO-Dia
Body weight (g)	31.56 ± 0.50	23.99 ± 0.20$^{\#\#\#}$	31.24 ± 0.69	23.37 ± 0.27$^{\#\#\#}$
Food intake (g/day)	3.28 ± 0.13	4.77 ± 0.11$^{\#}$	3.13 ± 0.12	4.68 ± 0.18$^{\#}$
GLU (mmol/L)	6.92 ± 0.41	24.33 ± 1.09$^{\#\#\#}$	8.61 ± 0.20	25.04 ± 0.63$^{\#\#\#}$
TG (mmol/L)	1.08 ± 0.08	2.24 ± 0.13$^{\#\#\#}$	0.68 ± 0.04**	0.79 ± 0.05***
TC (mmol/L)	2.06 ± 1.20	2.43 ± 1.16	2.32 ± 0.13	2.12 ± 0.10
FFA (mEq)	1.61 ± 0.24	1.96 ± 0.34	2.07 ± 0.09	1.94 ± 0.10
24 h UAE (μg/24 h)	28.35 ± 7.63	470.40 ± 104.00$^{\#}$	33.40 ± 8.77	364.10 ± 81.20$^{\#}$
24 h UCR (μmol/24 h)	5.94 ± 1.50	22.46 ± 1.43$^{\#\#\#}$	4.01 ± 1.11	11.79 ± 1.05$^{\#/***}$
Plasma creatinine (μmol/L)	42.28 ± 5.45	62.75 ± 8.25	35.91 ± 5.17	43.04 ± 7.11
Ccr (μL/min)	135.40 ± 33.79	224.7 ± 27.18$^{\#}$	111.70 ± 19.12	207.90 ± 41.55$^{\#}$
KW/BW (mg/g)	13.28 ± 0.64	20.41 ± 1.31$^{\#\#\#}$	12.85 ± 0.78	19.45 ± 1.27$^{\#}$
Glomerular surface area (μm^2)	6106 ± 221	9060 ± 200$^{\#\#\#}$	5739 ± 115	8951 ± 567$^{\#\#\#}$
Mesangial suface area (μm^2)	1087 ± 51	2012 ± 104$^{\#\#\#}$	1116 ± 52	1862 ± 108$^{\#\#\#}$

$^{**}p < 0.01$, $^{***}p < 0.001$ vs. WT mice, effect of genotype. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$ vs. ND mice, effect of diabetes. WT, wild-type; KO, ApoC3 knockout; ND, non-diabetic; Dia, diabetic; GLU, glucose; TG, triglyceride; TC, total cholesterol; FFA, free fatty acid; UAE, urinary albumin excretion; UCR, urinary creatinine; Ccr, creatinine clearance; KW/BW, kidney weight/body weight.

and similar to the results in the absence of HG conditions, TRLs-C3 elevated these parameters to a greater degree than TRLs-WT (Fig. 6A). The NF-κB inhibitor BAY11-7082 and TLR2 siRNA decreased HG+TRLs-C3-induced increases in the mRNA expression levels of α-SMA, Col IV, fibronectin, TGF-β and CTGF in HMCs (Fig. 6B–C).

4. Discussion

DN is a major microvascular complication of diabetes and an important cause of end-stage renal disease. Clinical studies suggest that ApoC3 is a risk factor for T1DN. We demonstrated for the first time that ApoC3 accelerated early-stage T1DN with increased renal inflammation in ApoC3 Tg diabetic mice. ApoC3 accelerated inflammation by activating the TLR2/NF-κB pathway in HMCs, which was partially independent of TG levels.

Diabetes is often accompanied by HTG [2,35], which is consistent with the increase in TG levels in STZ-induced diabetic mice observed in our study (Fig. 1D). HTG plays an important role in DN. TRLs could damage glomeruli and increase the permeability of the glomerular filtration barrier which may contribute to the progression of DN [36,37]; however, the exact mechanism was unclear. TRLs have been implicated in inducing inflammation via the NF-κB pathway in several kinds of cells, such as endothelial cells, monocytes and smooth muscle cells [38]. In this study, we demonstrated for the first time that TRLs activated the TLR2/NF-κB proinflammatory signaling pathway and increased expression levels of the downstream inflammatory factors TNF-α, VCAM-1 and MCP-1 in HMCs (Fig. 5B–G). In vivo, we found ApoC3 overexpression aggravating the development of T1DN in ApoC3 Tg mice exhibiting HTG, which exhibited plasma TG concentrations of 20–30 mmol/L (Fig. 1D). It is difficult to distinguish the effects of HTG and ApoC3 on T1DN in vivo. Therefore, we used TRLs with different ApoC3 contents at the same concentration to study whether the effects of ApoC3 on the development of DN are independent of HTG in vitro. We found TRLs with a high ApoC3 content had a greater effect than TRLs with a low ApoC3 content utilized at the same concentration (Fig. 5B–G), indicating that ApoC3 is involved in the effect of TRLs in accelerating inflammation. These findings suggest that ApoC3 aggravated T1DN-related inflammation in AopC3 Tg diabetic mice in a manner partially independent of HTG.

Tg-ND mice had approximately 20-fold higher plasma TG levels than WT-ND mice (Fig. 1D), which, unlike Tg-Dia mice, did not exhibit renal function injury or inflammation (Figs. 2–4). A previous study also showed that ApoC3 Tg mice with HTG had no kidney injury and that renal injury in these mice appeared only after severe acute pancreatitis [39]. These findings suggest that HTG may not be the main initiator of kidney injury but rather contributes to kidney injury under some pathologic conditions. In our study, we suspected that diabetes induced renal injury, after which TRLs permeated into the glomerulus and further increased renal inflammation. The results of the in vitro experiment also verified this hypothesis, as the incubation of HMCs with TRLs activated inflammation with or without HG conditions (Fig. 5).

C57BL/6 strain mice are relatively resistant to the development of DN. They exhibit mild to moderate albuminuria and mesangial matrix expansion even at 6 months after STZ-induced diabetes [40–42], and insulin treatment could alleviate the renal injury. To observe relatively obvious renal damage at 4 months after diabetes, insulin-treatment was not applied in this study as in the previous studies [43–45]. However, it would be interesting to determine the efficacy of insulin treatment for T1DN in ApoC3 Tg mice compared to WT mice in future work, as they share many similarities to the progression of T1DN in humans.

The transcriptional factor NF-κB is an important regulator of inflammation and plays a crucial role in DN [46,47]. ApoC3 could activate the NF-κB pathway in vascular endothelial cells, causing monocytes and macrophages to adhere to endothelial cells [24]. Our findings confirmed the activation of NF-κB in the kidney and HMCs under HG conditions, and importantly, ApoC3 overexpression further accelerated NF-κB activation in vivo and in vitro (Figs. 4A–C, 5D–G). The activation of inflammatory factors and transdifferentiation induced by TRLs-C3 in HMCs was effectively reduced by the inhibition of NF-κB with BAY11-7082 (Figs. 5D, E, H and 6B). These results indicated that NF-κB plays a primary role in ApoC3-induced inflammation in HMCs.

Consistent with previous studies, we found that HG conditions increased renal TLR2 expression levels in diabetic mice (Fig. 4A–C) [48]. TLR2 agonists have been shown to accelerate kidney injury, whereas TLR2 deficiency ameliorated renal hypertrophy and albuminuria in diabetic mice [25]. These results suggest that elevated TLR2 expression levels in the kidney may contribute to the progression of DN. Several studies have emphasized the role of TLR2 in orchestrating inflammation in DN [25]. TLR2 induces the activation of NF-κB, resulting in renal injury in DN [3]. A study showed that the activation of NF-κB was prevented by TLR2 silencing, and this study emphasized the importance of TLR2 as a mediator of NF-κB activation in DN [49]. Interestingly, TLR2 expression levels were decreased after NF-κB inhibition (Fig. 5F, G and I), which is consistent with previous studies [50,51]. TLR2 could activate NF-κB, and activated NF-κB could promote the expression of TLR2 transcriptionally, leading to a TLR2-NF-κB-TLR2 positive feedback loop that induced the inflammatory response [52]. This might explain the decrease in TLR2 expression after NF-κB inhibition. ApoC3 is an endogenous ligand of TLR2. In skeletal muscle cells, ApoC3 activated TLR2 to induce the activation of NF-κB pathway-related inflammation and insulin resistance [23]. In our study, both high ApoC3 expression in vivo and in vitro contributed to activation of the TLR2/NF-κB pathway and increased the expression levels of downstream inflammatory factors (Figs. 4–5). ApoC3-induced activation of the NF-κB pathway and transdifferentiation were suppressed by the

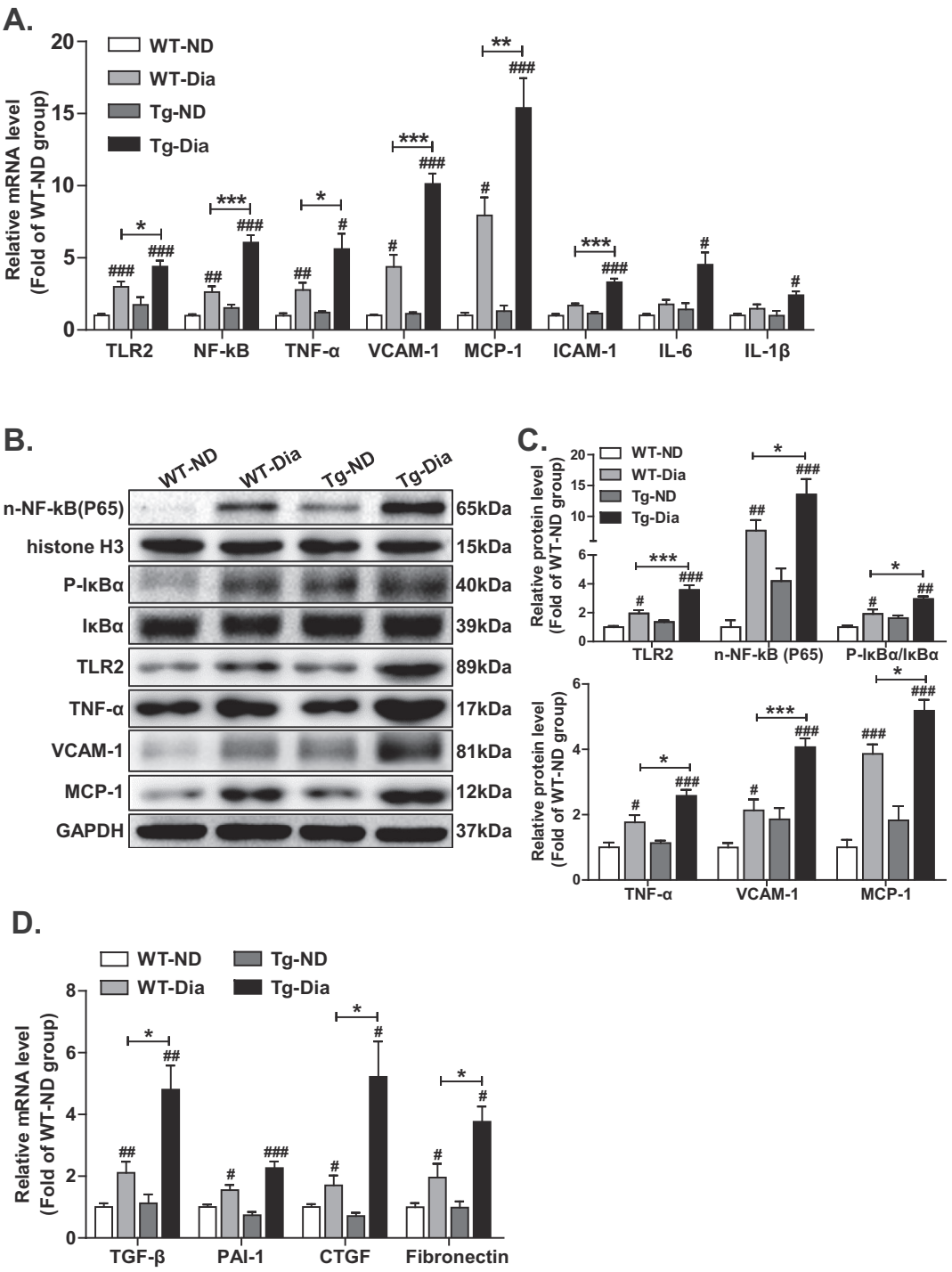

Fig. 4. Renal TLR2/NF-κB pathway-related gene and protein expression levels in WT and Tg mice under diabetic conditions. (A) Renal gene expression levels of TLR2, NF-κB, TNF-α, VCAM-1, MCP-1, ICAM-1, IL-6 and IL-1β. (B) Representative Western blot images showing n-NF-κB (p65), histone H3, P-IκBα, IκBα, TLR2, TNF-α, VCAM-1, MCP-1 and GAPDH levels in the kidney. (C) Quantification of TLR2, n-NF-κB (p65), the P-IκBα/IκBα ratio, TNF-α, VCAM-1 and MCP-1 in the kidney by densitometry. (D) Expression levels of the fibrogenesis-related genes PAI-1, TGF-β, CTGF and fibronectin. All data were collected at 4 months after diabetes. n = 8–10 mice per group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. WT mice, effect of genotype. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. ND mice, effect of diabetes. WT, wild-type mice; Tg, ApoC3 transgenic mice; ND, non-diabetes; Dia, diabetes.

blockade of TLR2 with siRNA in HMCs (Figs. 5F, G, I and 6C). These data suggest that TLR2 plays an important role in ApoC3-induced inflammation in HMCs.

A recent study identified ApoC3 in TRLs as a mediator leading to alternative inflammasome activation in human monocytes by the dimerization of TLR2 and TLR4 [53]. The authors found that blocking TLR2 or TLR4 decreased ApoC3-induced inflammasome-dependent IL-1β production in human monocytes. Although the expression of TLR2 is higher than that of TLR4 in glomeruli, TLR4 also plays a role in DN [54]. Therefore, whether TLR4 is involved in ApoC3-induced NF-κB pathway-mediated inflammation in DN and whether ApoC3 directly binds TLR2 or TLR4 need further investigation.

Tg-Dia mice showed increased renal mesangial surface areas and mesangial lipid deposition (Fig. 3b–d). Mesangial cells are one of the

major matrix-producing cell types. Increased TLR2 expression induces MCP1 production in mesangial cells [27]. Therefore, we used HMCs, which are closer to human cells, for in vitro studies. ApoC3 is mainly expressed in the liver and small intestine and hardly expressed in the kidney. In circulation, ApoC3 mainly resides on the surface of TRLs, and unbound ApoC3 is quickly degraded. Therefore, we used TRLs

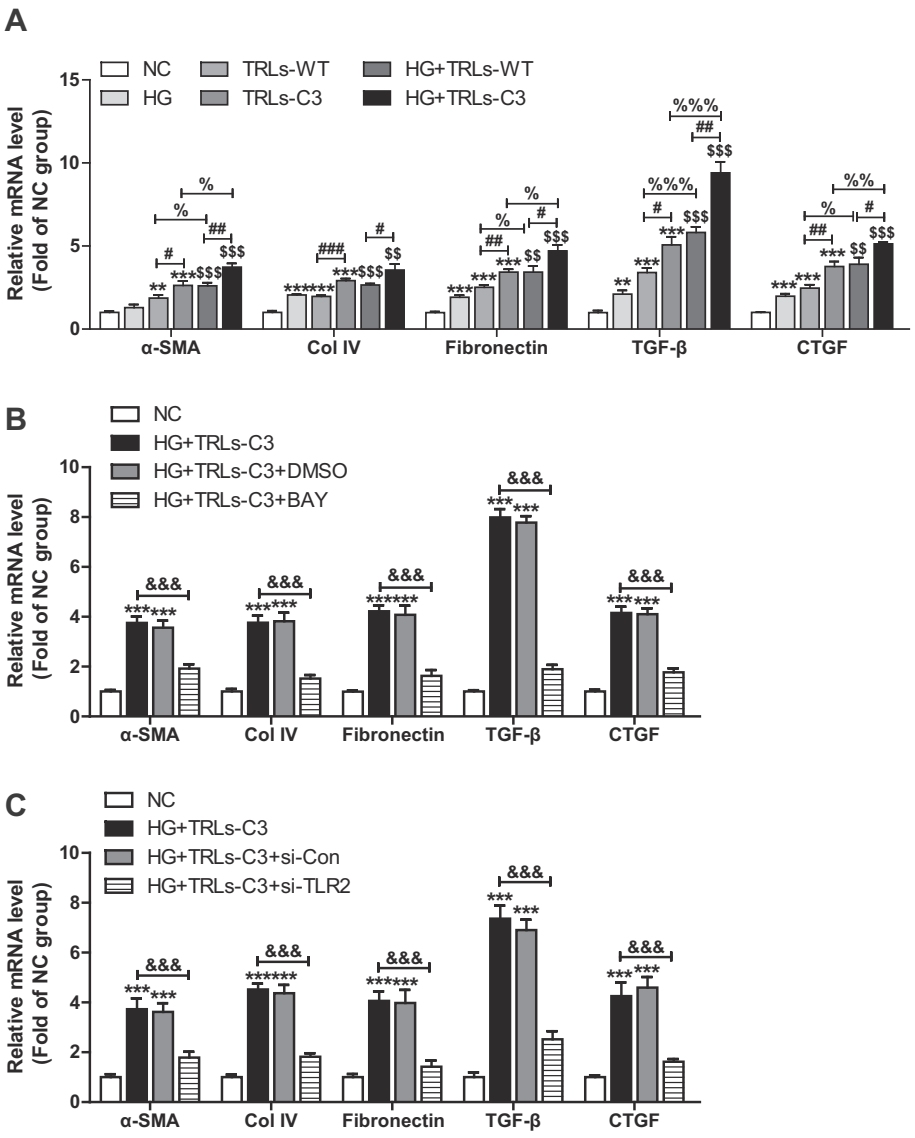

Fig. 6. Fibrogenesis-related gene expression levels in HMCs treated with TRLs with/without HG conditions. Gene expression levels of α-SMA, Col IV, Fibronectin, TGF-β and CTGF in HMCs treated with HG+TRLs (A), HG+TRLs-C3+BAY (B) or HG+TRLs-C3+si-TLR2 (C). $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the NC group, effect of TRLs under normal-glucose conditions; $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs. the HG group, effect of TRLs under HG conditions; $^{\%}p < 0.05$, $^{\%\%}p < 0.01$, $^{\%\%\%}p < 0.001$ vs. TLRs-WT or TRLs-C3, effect of HG; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. TLRs-WT at the same concentration, effect of ApoC3; $^{\&\&\&}p < 0.001$ vs. HG+TLRs-C3, effect of BAY or si-TLR2. TRLs-C3, ApoC3 transgenic mouse-derived TRLs; TRLs-WT, WT mouse-derived TRLs; NC, normal control; HG, high glucose; BAY, BAY11-7082; si-TLR2, TLR2 siRNA; si-Con, control siRNA.

with different levels of ApoC3, not ApoC3 alone as previously described [7], to observe the effect of ApoC3 on HMCs which imitates functions of ApoC3 in vivo. We speculated that ApoC3 bound to TRLs in the circulation may be exposed to mesangial cells through the damaged vascular endothelium, thereby activating TLR2/NF-κB pathway-related inflammation in mesangial cells and aggravating T1DN. Activated TLR2/NF-κB in renal endothelial cells and podocytes also plays a role in renal injury [3,25]. Whether ApoC3 affects TLR2/NF-κB pathway-related inflammation in renal endothelial cells and podocytes should be further investigated.

Unfortunately, we did not find that ApoC3 deficiency had an obvious protective effect against DN in STZ-induced mice (Table 1). Similar to the results of another study, overexpression of human ApoC3 in mice aggravated restenosis and atherogenesis by promoting the proliferation of smooth muscle cells, but ApoC3 deficiency was not found to have a protective effect in mice [7]. Human and mouse ApoC3 share only

Fig. 5. ApoC3 protein expression in TRLs and activation of the TLR2/NF-κB pathway and expression of downstream proinflammatory cytokines in HMCs stimulated with TRLs with/without HG conditions. (A) Representative and the quantification of Western blot of ApoC3 in TRLs. Gene expression levels of NF-κB (p65), TNF-α and VCAM-1 in HMCs treated with TRLs (B) or HG+TRLs (C). Representative and the quantification of Western blot of n-NF-κB (p65), P-IκBα/IκBα, TLR2, TNF-α, VCAM-1, MCP-1 and the loading controls histone H3 and GAPDH in HMCs treated with BAY (D, E) or si-TLR2 (F, G) with TRLs under HG conditions. Gene expression levels of TLR2, NF-κB (p65), TNF-α, VCAM-1, MCP-1, ICAM-1 and IL-6 in HMCs treated with BAY (H) or si-TLR2 (I) with TRLs-C3 under HG conditions. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. the NC or 0.2 mg/mL TRLs group, effect of TRLs under normal-glucose conditions; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ vs. the HG group, effect of TRLs under HG conditions; $^{\%}p < 0.05$, $^{\%\%}p < 0.01$, $^{\%\%\%}p < 0.001$ vs. TLRs-WT or TRLs-C3, effect of HG; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. TLRs-WT at the same concentration, effect of ApoC3; $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ vs. HG+TLRs-C3, effect of BAY or si-TLR2. TRLs, triglyceride-rich lipoproteins; TRLs-C3, ApoC3 transgenic mouse-derived TRLs; TRLs-WT, WT mouse-derived TRLs; NC, normal control; HG, high glucose; MA, mannitol control; BAY, BAY11-7082; si-TLR2, TLR2 siRNA; si-Con, control siRNA.

62.9% homology [53]. Due to differences in their structural and functional properties, mouse and human ApoC3 may significantly impact DN to different extents. We tried to investigate the effect of TRLs without ApoC3 in HMCs. However, it was difficult to isolate TRLs from ApoC3 KO mice due to the low levels of TG and TRLs, as shown in previous studies [7,55]. In addition, lipid metabolism significantly differs between mice and humans [56]. An animal model of lipid metabolism that mimics lipid metabolism in humans is needed to investigate the effects of ApoC3 deficiency in DN.

Clinical studies have shown that plasma ApoC3 levels are increased in T1DN patients [20,22]. Our study found that high plasma ApoC3 levels aggravated the development of T1DN in mice. Volanesorsen, an ASO of ApoC3, has actually been applied to patients with familial chylomicronemia syndrome. The determination of the efficacy of volanesorsen for the treatment of T1DN with high plasma ApoC3 and TG levels requires more animal experiments and clinical investigations. Mice expressing different levels of human ApoC3 which approximate to that in human T1DN should also be study in further research.

5. Conclusions

In summary, ApoC3 overexpression aggravated the development of early-stage TIDN with increased renal inflammation in Tg diabetic mice. ApoC3 accelerated inflammation by activating the TLR2/NF-κB pathway in HG-stimulated HMCs, and these effects were inhibited by the blockade of TLR2 or NF-κB and were partially independent of TG levels.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2021.154740.

Funding

This work was supported by the grant from National Natural Science Foundation of China [grant numbers 81770448 and 81470553] awarded to WH and Beijing Natural Science Foundation [grant numbers 7204248] awarded to HW.

CRediT authorship contribution statement

Huan Wang: Investigation, Data curation, Writing – original draft, Visualization, Project administration. **Xiaomin Huang:** Investigation, Validation, Data curation. **Pengfei Xu:** Investigation, Formal analysis. **Xuejing Liu:** Investigation. **Zihao Zhou:** Investigation. **Fuhua Wang:** Investigation. **Jingyi Li:** Investigation. **Yuhui Wang:** Methodology. **Xunde Xian:** Writing – review & editing. **George Liu:** Writing – review & editing. **Wei Huang:** Supervision, Conceptualization, Writing – review & editing, Funding acquisition.

Declaration of competing interest

None.

Acknowledgements

The authors would like to thank Professor Youfei Guan (Advanced Institute for Medical Sciences, Dalian Medical University) for providing HMCs.

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