BBA - Molecular Basis of Disease 1865 (2019) 1332–1340

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis

Lysophosphatidic acid receptor 1 inhibitor, AM095, attenuates diabetic nephropathy in mice by downregulation of TLR4/NF-κB signaling and NADPH oxidase

Jong Han Lee[a,b], Mithun Kumer Sarker[a,b], Hojung Choi[a,b], Dongyun Shin[a], Donghee Kim[b], Hee-Sook Jun[a,b,c,*]

[a] College of Pharmacy, Gachon University, Incheon, Republic of Korea
[b] Lee Gil Ya Cancer and Diabetes Institute, Gachon University, Incheon, Republic of Korea
[c] Gachon University Gil Medical Center, Gachon Medical and Convergence Institute, Incheon, Republic of Korea

ARTICLE INFO

Keywords:
Diabetic nephropathy
Lysophosphatidic acid
Lysophosphatidic acid receptor 1
AM095
Reactive oxidative stress
Inflammatory cytokines

ABSTRACT

Diabetic nephropathy (DN) is one of the major long-term complications of diabetes. Lysophosphatidic acid (LPA) signaling has been implicated in renal fibrosis. In our previous study, we found that the LPA receptor 1/3 (LPAR1/3) antagonist, ki16425, protected against DN in diabetic *db/db* mice. Here, we investigated the effects of a specific pharmacological inhibitor of LPA receptor 1 (LPA1), AM095, on DN in streptozotocin (STZ)-induced diabetic mice to exclude a possible contribution of LPAR3 inhibition. AM095 treatment significantly reduced albuminuria and the albumin to creatinine ratio and significantly decreased the glomerular volume and tuft area in the treated group compared with the STZ-vehicle group. In the kidney of STZ-induced diabetic mice, the expression of LPAR1 mRNA and protein was positively correlated with oxidative stress. AM095 treatment inhibited LPA-induced reactive oxygen species production and NADPH oxidase expression as well as LPA-induced toll like receptor 4 (TLR4) expression in mesangial cells and in the kidney of STZ-induced diabetic mice. In addition, AM095 treatment suppressed LPA-induced pro-inflammatory cytokines and fibrotic factors expression through downregulation of phosphorylated NFκBp65 and c-Jun N-terminal kinases (JNK) in vitro and in the kidney of STZ-induced diabetic mice. Pharmacological or siRNA inhibition of TLR4 and NADPH oxidase mimicked the effects of AM095 in vitro. In conclusion, AM095 is effective in preventing the pathogenesis of DN by inhibiting TLR4/NF-κB and the NADPH oxidase system, consequently inhibiting the inflammatory signaling cascade in renal tissue of diabetic mice, suggesting that LPAR1 antagonism might provide a potential therapeutic target for DN.

1. Introduction

Diabetic nephropathy (DN) is the major microvascular complication of diabetes [1]. Around 30% of diabetic patients finally progress to end-stage renal disease [2,3], which is characterized by glomerulosclerosis and inter-capillary glomerulonephritis with micro/macro-albuminuria [4,5]. Inflammation and oxidative stress are known as the major causes for the development of DN [6].

Inflammatory cytokines, including interleukin 6 (IL6), tumor necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β1) are elevated in plasma of patients with kidney disease [7]. Kidney fibrosis is mostly preceded by and closely associated with chronic interstitial inflammation [7–9]. TGF-β1 is the primary factor that promotes fibrosis in chronic kidney disease [10–13]. The activation of TGF-β1 or its downstream signaling pathways promotes extracellular matrix expansion leading to thickening of the basement membrane and glomerulosclerosis in the kidney [13].

Toll like receptors (TLRs) mediate innate immune and inflammatory responses [14–17]. TLR4 is an innate immune receptor classically activated by lipopolysaccharide in immune cells or by endogenous ligands (such as oxidized lipoproteins) in nonimmune cells [18,19]. TLR4 activates nuclear factor-κB (NF-κB), the master transcription factor controlling inflammatory genes. TLR4 expression increases in various cell types and organs of diabetic patients [16,20]. Some studies have

Abbreviations: DN, diabetic nephropathy; LPA, lysophosphatidic acid; LPAR, LPA receptor; STZ, streptozotocin; TLR4, toll like receptor 4; JNK, c-Jun N-terminal kinases

* Corresponding author at: College of Pharmacy, Gachon University, 191 Hambakmoero, Yeonsu-Gu, Incheon 21936, Republic of Korea.
E-mail address: hsjun@gachon.ac.kr (H.-S. Jun).

https://doi.org/10.1016/j.bbadis.2019.02.001
Received 10 October 2018; Received in revised form 15 January 2019; Accepted 4 February 2019
Available online 11 February 2019

indicated that stress-activated protein kinases of the mitogen activated protein kinase (MAPK) family, such as c-Jun N-terminal kinase (JNK) and p38, are associated with DN in human and rodent models [21]. Therefore, the activation of NF-κB and MAPK may contribute to pathogenesis of DN [22,23].

Lysophosphatidic acid (LPA) is a small phospholipid derivative that acts as a potent mitogen through its G protein coupled receptors (LPAR1-6s) [24]. The release of LPA mediates pro-inflammatory responses in several tissues including the kidney [25,26]. Recent studies have also shown that LPA is increased in the serum of human diabetic patients and kidney cortex of *db/db* mice [27,28], implicating it in diabetic kidney diseases. AM095 is an antagonist of the LPA type 1 receptor [29]. It has been shown to inhibit LPA-induced chemotaxis in both mouse LPA1R over-expressed CHO cell lines and human A2058 melanoma cells. In addition, AM095 suppresses LPA-induced histamine release and reduces macrophage and lymphocyte infiltration in mice administered with bleomycin [30]. These findings suggest that AM095 may be a potential therapeutic antagonist for blocking abnormally activated LPA signaling in the hyperglycemia condition, thereby reducing the inflammatory response of DN. However, it is not clear whether AM095 is effective in DN.

In this study, we investigated the effects of LPA receptor 1 antagonist (AM095) in a streptozotocin (STZ)-induced mouse model of type 1 diabetes and further determined the underlying molecular mechanisms.

2. Materials and methods

2.1. Animals

Eight-week-old C57BL/6J male mice (n = 50) were used for the induction of type 1 diabetes by STZ treatment, as described elsewhere [31]. Briefly, mice were treated with 50 mg/kg of STZ i.p. daily (Sigma, S0130) after 4 h (h) fasting in the morning for 5 days. Diabetes was confirmed by blood glucose levels > 350 mg/dl. STZ-diabetic mice with similar body weights were then randomly divided into: STZ-vehicle group, STZ-AM095 treated group, and STZ-losartan treated group. Age-matched non-diabetic C57BL/6J male mice were used as the control vehicle group. AM095 was orally injected every day in the morning at a dosage of 10 mg/kg or 30 mg/kg AM095, or 10 mg/kg losartan (Sigma, 61188) for 8 weeks. All procedures were approved by the Institutional Animal Care and Use Committee at Gachon University.

2.2. Biochemical parameters of blood and urine

Mice were placed in individual mouse metabolic cages for 24 h after 4 weeks and 8 weeks of administration with AM095 or losartan. Food intake, water intake, and urine volumes were monitored. Hemoglobin A1c (HbA1c) was determined from blood using a DCA System HbA1c Reagent Kit (SIEMENS, New York, USA). Blood glucose from the tail vein blood was measured using a glucose analyzer (One Touch®Ultra, Lifescan Johnson & Johnson, Milpitas, CA) after 4 h of fasting in the morning. The levels of cholesterol and triglyceride were assessed using commercially available kits (ASAN HDL-Cholesterol, # AM203, ASAN; TG-s, #AM157K, ASAN) according to manufacturer's instructions. Urine was also collected during metabolic cage study for 24 h. All parameters, such as creatinine and micro-albumin, were measured using a biochemical analyzer (Beckman, USA).

2.3. Renal histological assessment

The mice were sacrificed, and the kidneys were harvested. The right kidney was rapidly fixed in 10% formalin buffer and embedded in paraffin. Sections were stained as described previously [32] with hematoxylin and eosin or periodic acid-Schiff, or with antibodies for 4-hydroxynonenal (4-HNE) (Abcam, #ab46545) and fibronectin. Antibodies were visualized by DAB substrate chromogen system (DAKO, K346811) under light microscopy.

2.4. Cell culture

SV40 MES cells, a mouse mesangial cell line, were obtained from American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium containing 5% fetal bovine serum and 1% penicillin-streptomycin. To investigate the effect of LPA (Avanti Polar Lipids, Alabaster, Alabama), the cells were seeded and pretreated with 0.1% fatty acid free-bovine serum albumin for 12–16 h, then treated with 5 μM of LPA or 10 μM of various inhibitors (TLR4-IN-C34 for TLR4; VAS2870 for NADPH oxidase; Sigma, St. Louis, MO) 1 h prior to LPA treatment.

2.5. ROS measurement

To assess the reactive oxidative species (ROS) caused by LPA, we measured ROS production levels after incubation with 5 μM of dichlorofluorescein for 30 min in the dark using flow cytometry (up to 1×10^4 cells) with a FACS LSRII flow cytometer equipped CellQuest™ Pro Software (BD Bioscience, San Jose, CA).

2.6. Transfection

SV40 MES13 cells were seeded in 6 well plates with 5×10^4 cells/well, and then, transiently transfected with siRNA control (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and siRNA TLR4 using Lipofectamine RNAiMAX (Invitrogen) reagent as per the manufacturer's instructions. After 36 h, the medium was replaced with serum-free medium containing 0.1% fatty acid-free bovine serum albumin for 12–16 h, and the cells were treated with LPA (5 μM) for 3 h.

2.7. Western blot

Total protein was isolated using mammalian protein extract buffer (GE Life Science, 28-9712-79) containing protease inhibitor cocktail (Sigma, P8340). Signals were detected using an enhanced chemiluminescence detection system (Millipore, Watford, UK). The band density was quantified using the *Image J* program and normalized by actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The antibodies used were as follows: anti-actin (Cell Signaling, #8457), anti-GAPDH (Millipore, #MAB374), anti-p-NFκB p65 (Ser536) (Cell Signaling, #3033), anti-NF-κB (Santa Cruz, sc-372), anti-p-stress activated protein kinase (SAPK)/JNK (Cell Signaling, #9251), anti-SAPK/JNK (Cell Signaling, #9252), anti-LPAR1 (Abcam, #ab23698), anti-fibronectin (Santa Cruz, sc-8422), anti-gp91-phox (Santa Cruz, sc-130543), anti-TLR4 (Santa Cruz, sc-293072), anti-collagen 1 type 1 (CoL1A1; Santa Cruz, sc-293182), anti-extracellular signal–regulated kinase 1/2 (Erk 1/2; Cell Signaling, #9102S), anti-p-Erk 1/2 (Cell Signaling, #9101S), and anti-tissue inhibitor of metalloproteinase-1 (TIMP1; Santa Cruz, sc-21734).

2.8. Reverse transcriptase-quantitative polymerase chain reaction

Total RNA was isolated from kidney tissue of STZ-induced diabetic mice or mesangial cells using RNAiso (Takara) reagent. The cDNA was synthesized from 2 μg total RNA using the PrimeScript 1st strand cDNA synthesis kit (TAKARA, 6110A). Quantitative real-time PCR was performed using Applied-Biosystem Prism 7900HT Real-Time PCR. The relative gene expression levels were normalized by cyclophilin A RNA expression. The primers used are listed in Supplementary Table 1.

2.9. Statistical analysis

All results were expressed as the mean ± SEM. All in vitro experiments were conducted at least independent three times and in vivo

experiment were performed as indicated in legends. Differences among more than two groups were analyzed by one ANOVA followed by Tukey's post hoc multiple comparison tests. An unpaired 2-tailed *t*-test was used for analysis of two groups. Significance was determined if p values were < 0.05.

3. Results

3.1. AM095 treatment attenuates hyperglycemia and dyslipidemia in STZ-induced diabetic mice

We investigated the effects of AM095, a specific LPAR1 antagonist, on DN in STZ-induced diabetic mice. STZ-induced diabetic mice were given either AM095 (10 or 30 mg/kg), vehicle, or losartan daily for 8 weeks. Losartan is an angiotensin II type 1 receptor antagonist used to reduce the progression of renal disease and was used here as a positive control. Body weights among the STZ groups were comparable after 8 weeks of treatment (Table 1). Triglyceride level was significantly reduced in STZ-30 mg AM095 mice compared with STZ-vehicle mice, but cholesterol level was not changed (Supplementary Fig. 1). Blood glucose and HbA1c were highly increased by STZ but significantly, although mild, lowered in the two STZ-AM095-treated groups compared with the STZ-vehicle group (Table 1). The losartan group showed effects similar to AM095 group (Table 1).

3.2. AM095 treatment improves kidney function in STZ-induced diabetic mice

In order to evaluate whether AM095 improves kidney function, we monitored food and water consumption, as well as urine volume. Food intake, water intake, and urine volume were increased by STZ, and these increases were ameliorated by 30 mg AM095 or losartan (Table 1). Moreover, albumin in the urine and the albumin/creatinine ratio were increased in STZ-induced diabetic mice compared with the vehicle control group; but only the albumin/creatinine ratio was significantly decreased by 30 mg AM095 or losartan administration (Table 1).

3.3. AM095 treatment reduces glomerular injury in the kidney of STZ-induced diabetic mice

Glomerular matrix expansion is a hallmark of DN in kidney [32], therefore we investigated glomerular structural alterations in kidney tissue by hematoxylin and eosin or Periodic Acid–Schiff staining (Fig. 1A). The glomerular surface area and glomerular tuft area were significantly increased in diabetic mice, and glomerular surface area was normalized by 30 mg AM095 or losartan treatment (Fig. 1B, C). Since blood urea nitrogen and creatinine are biomarkers for renal function, we also measured their levels in serum after 8 weeks of AM095 treatment. These two parameters were higher in the STZ-vehicle group; however, these rises were significantly reduced by 30 mg AM095 or losartan, suggesting that AM095 recovers renal function (Fig. 1D, E).

3.4. The increase of LPAR1 expression is correlated with increase of oxidative stress in the kidney of STZ-induced diabetic mice

To investigate the possibility of oxidative stress as a causative intermediary of LPA signaling in diabetic kidney failure, we first assessed whether LPA signaling is activated in the STZ-diabetic model. In the kidney tissue of STZ-induced diabetic mice, LPAR1 mRNA levels significantly increased but the levels of mRNAs of LPAR2 and LPAR3 were similar with those in the non-diabetic model (Fig. 2A, B, C). Consistently, LPAR1 protein expression was also significantly increased (Fig. 2D). Since the lipid-aldehyde, 4-HNE, is one of the primary by-products of lipid peroxidation under oxidative stress, it is considered to be an oxidative stress marker [33]. We found that 4-HNE immunofluorescence in the diabetic condition was remarkably higher than that of the vehicle control mice (Fig. 2E).

3.5. AM095 treatment suppresses ROS production by down-regulating NADPH oxidase and TLR4 protein expression in mesangial cells treated with LPA and in diabetic mice

To investigate whether AM095 suppresses LPA-mediated ROS production, we measured ROS production in LPA-treated SV40 MES13 cells, a mouse mesangial cell line, using flow cytometry. We found that LPA treatment increased ROS production, whereas AM095 treatment significantly inhibited this increase by around 50% compared with that in LPA only-treated cells (Fig. 3A, B). Consistently, LPA-mediated ROS production was suppressed in cells pretreated with TLR4 inhibitor (TLR4-IN-C34) (Supplementary Fig. 2). Considering that NADPH oxidase is implicated in cellular ROS production [34], we investigated whether LPA activates NADPH oxidase. NADPH oxidase protein expression level increased in response to LPA, whereas AM095 attenuated this rise in mesangial cells (Fig. 3C). Similarly, NADPH oxidase mRNA (Fig. 3D) and protein (Fig. 3E) expressions were increased in the kidney of diabetic mice and were decreased by AM095 treatment. TLR4 activates the inflammatory response and simultaneously activates NADPH oxidase, which is the main pathway for ROS production [34]. Thus, we next investigated whether LPA directly induces TLR4 expression in SV40 MES13 cells. LPA increased TLR4 protein expression, which was reduced by AM095 treatment (Fig. 3F). In addition, TLR4 mRNA (Fig. 3G) and protein (Fig. 3H) expressions were increased in diabetic mice and reduced in AM095-treated mice, particularly at 30 mg of AM095.

Table 1
Effect of AM095 treatment on physiological parameters of STZ-induced diabetic mice.

Characteristic	CV	SV	S10A	S30A	S10L
Body weight (g)	29.1 ± 0.5	25.5 ± 0.7 †	25.8 ± 0.5	25.7 ± 0.5	26.0 ± 0.6
Kidney weight (g)	0.31 ± 0.006	0.37 ± 0.005†	0.37 ± 0.02	0.35 ± 0.007§	0.35 ± 0.008§
HbA1C (%)	4.37 ± 0.05	9.62 ± 0.19†	8.87 ± 0.29§	8.68 ± 0.30§	8.52 ± 0.35§
Blood glucose (mg/dl)	139 ± 5.04	430.77 ± 12.92†	354.28 ± 17.91§	341.77 ± 15.23‡	339.08 ± 28.17§
Food intake (g/g per day)	0.15 ± 0.01	0.32 ± 0.02†	0.30 ± 0.02 $^{p=0.09}$	0.29 ± 0.02§	0.27 ± 0.02§
Water intake (ml/g per day)	0.14 ± 0.01	0.74 ± 0.08†	0.70 ± 0.09	0.48 ± 0.06§	0.49 ± 0.07§
Urine volume (ml/g per day)	0.03 ± 0.004	0.67 ± 0.08†	0.49 ± 0.07	0.39 ± 0.07‡	0.34 ± 0.08‡
24 h albuminuria (mg)	1.18 ± 0.180	4.09 ± 0.289†	3.67 ± 0.339	3.23 ± 0.324$^{p=0.09}$	3.14 ± 0.429
Albumin/creatinine ratio (μg/mg)	0.028 ± 0.003	0.065 ± 0.008†	0.046 ± 0.005$^{p=0.08}$	0.041 ± 0.005§	0.037 ± 0.007§

Albuminuria, albumin/creatinine ratio at 4 weeks; other parameters collected at 8 weeks, HbA1c: glycosylated hemoglobin. Data are shown mean ± SEM; CV: control-vehicle SV: STZ-vehicle S10A: STZ-10 mg AM095 S30A: STZ-30 mg AM095. S10L: STZ-10 mg Losartan. $^{\dagger}p < 0.01$ vs. CV; $^{\ddagger}p < 0.01$ vs. SV; $^{\S}p < 0.05$ vs. SV, The noted p value vs. SV (n = 10).

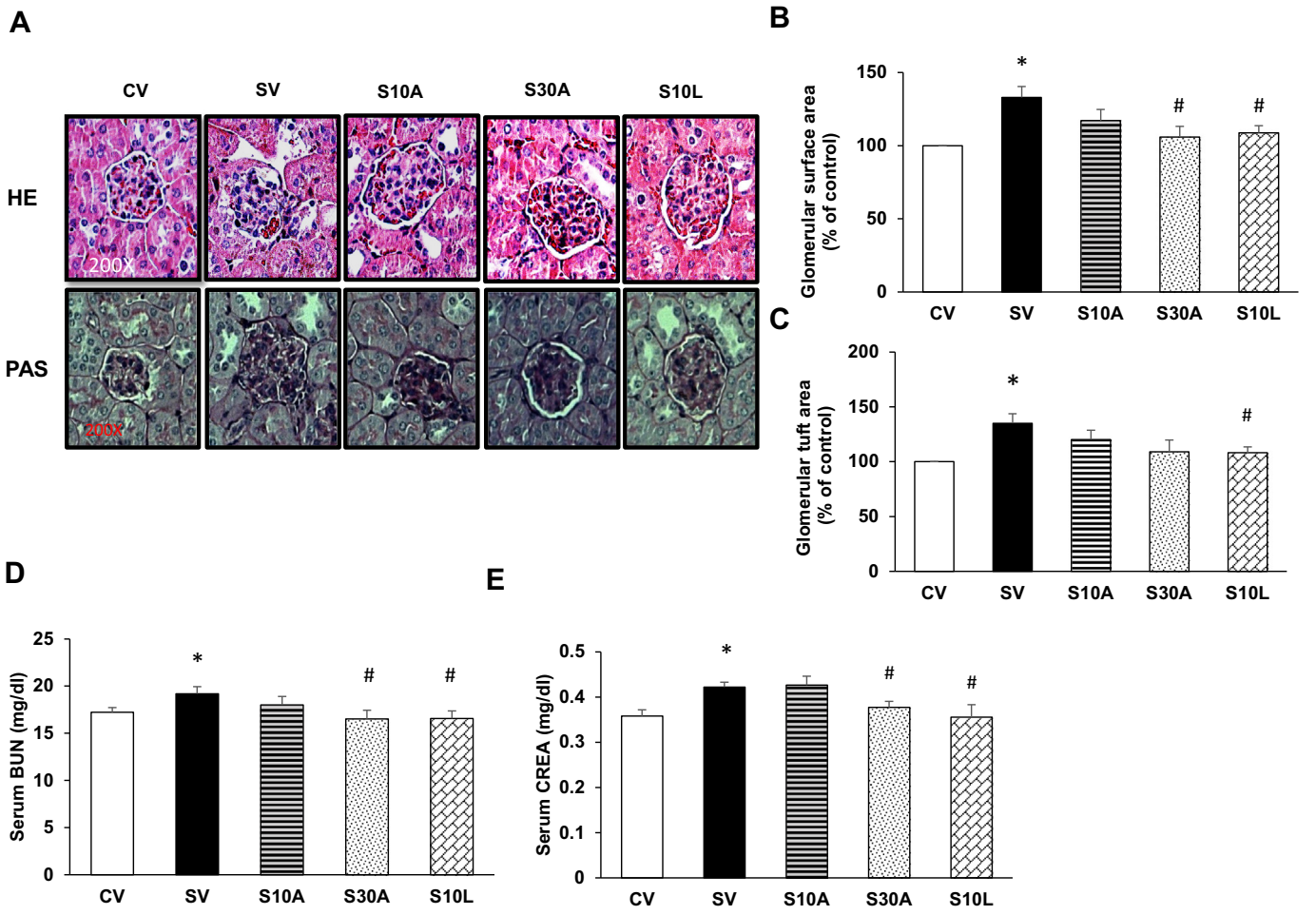

Fig. 1. AM095 treatment reduces glomerular injury in the kidney of STZ-induced diabetic mice. STZ-induced diabetic mice were administered either vehicle, AM095 (10 or 30 mg/kg orally), or losartan (10 mg/kg orally) for 8 weeks and then sacrificed. Non-diabetic mice were administered with vehicle as a control. (A) Representative image of renal tissue stained with hematoxylin and eosin (HE) or periodic acid-Schiff (PAS) for evaluation of mesangial matrix expansion. (B) Glomerular surface area and (C) glomerular tuft area were quantified using 30 glomeruli per mouse using *ImageJ* Software (n = 5) (D) The level of serum blood urea nitrogen (BUN) and (E) serum creatinine (CREA) were measured (n = 10–15); CV: control-vehicle; SV: STZ-vehicle; S10A: STZ-10 mg AM095; S30A: STZ-30 mg AM095; S10L: STZ-10 mg Losartan. *p < 0.05 vs. CV; #p < 0.05 vs. SV.

3.6. AM095 treatment suppresses the expression of pro-inflammatory cytokines and fibrotic factors through NF-κB and JNK signaling pathways in mesangial cells treated with LPA

We further assessed whether LPA induces pro-inflammatory cytokines and if this induction is inhibited by AM095 in vitro. The expressions of TNFα, IL1β, IL6, monocyte chemoattractant protein-1 (MCP-1), and TGFβ1 were significantly increased by LPA, and these increases were significantly ameliorated in the presence of AM095, except for IL6 and MCP1, which did not reach significance (Fig. 4A–E). LPA treatment increased phosphorylation of NFκBp65 (p65) and JNK, and AM095 suppressed their activation (Fig. 4F, G). In contrast, phosphorylated ERK expression was comparable among all experimental conditions (Fig. 4G, Supplementary Fig. 3).

3.7. LPA-induced expression of cytokines and fibrotic factors are mediated by TLR4 and NADPH oxidase

Our data from in vitro studies suggest that AM095 suppresses LPA-induced TLR4 expression as well as NADPH oxidase expression. Therefore, we determined whether the expression of inflammatory cytokines and fibrotic factors is inhibited by TLR4 or NADPH oxidase inhibition in mesangial cells. LPA-induced expressions of TNFα and TGFβ1 were inhibited in the presence of TLR4-IN-C34, a TLR4 inhibitor, and the expressions of TNFα, IL6, MCP1, and TGFβ1 were significantly inhibited by VAS2870, an NADPH oxidase inhibitor (Fig. 4H–K). None of the inhibitors suppressed the LPA-induced increase in fibronectin levels (Fig. 4L). Moreover, TLR4 knock-down using siRNA technique also significantly suppressed LPA-induced inflammatory cytokines and fibrotic factors expression compared with control siRNA transfected cells (Supplementary Fig. 4). Taken together, this inhibition pattern is similar to the effect of AM095 on SV40 MES13 cells exposed to LPA, suggesting that these two signaling pathways are a potential target of AM095.

3.8. AM095 treatment inhibits the expression of pro-inflammatory cytokines and fibrotic factors in the kidney of STZ-induced diabetic mice

To examine whether AM095 treatment affects the expression of pro-inflammatory cytokines and fibrotic factors in the kidney of STZ-induced diabetic mice, we checked the mRNA expression of inflammatory cytokines and fibrotic factors. The expressions of TNFα and IL1β were not increased by STZ treatment; however, IL6 was upregulated. In contrast, the expressions of TNFα, IL1β, and IL6 were significantly suppressed by 30 mg of AM095 (Fig. 5A–C). Interestingly, MCP1 expression levels were low in all the STZ-induced diabetic groups compared with the control-vehicle mice (Fig. 5D). TGFβ1 is well known for upregulating fibrotic factors [32]. TGFβ1 and fibronectin were

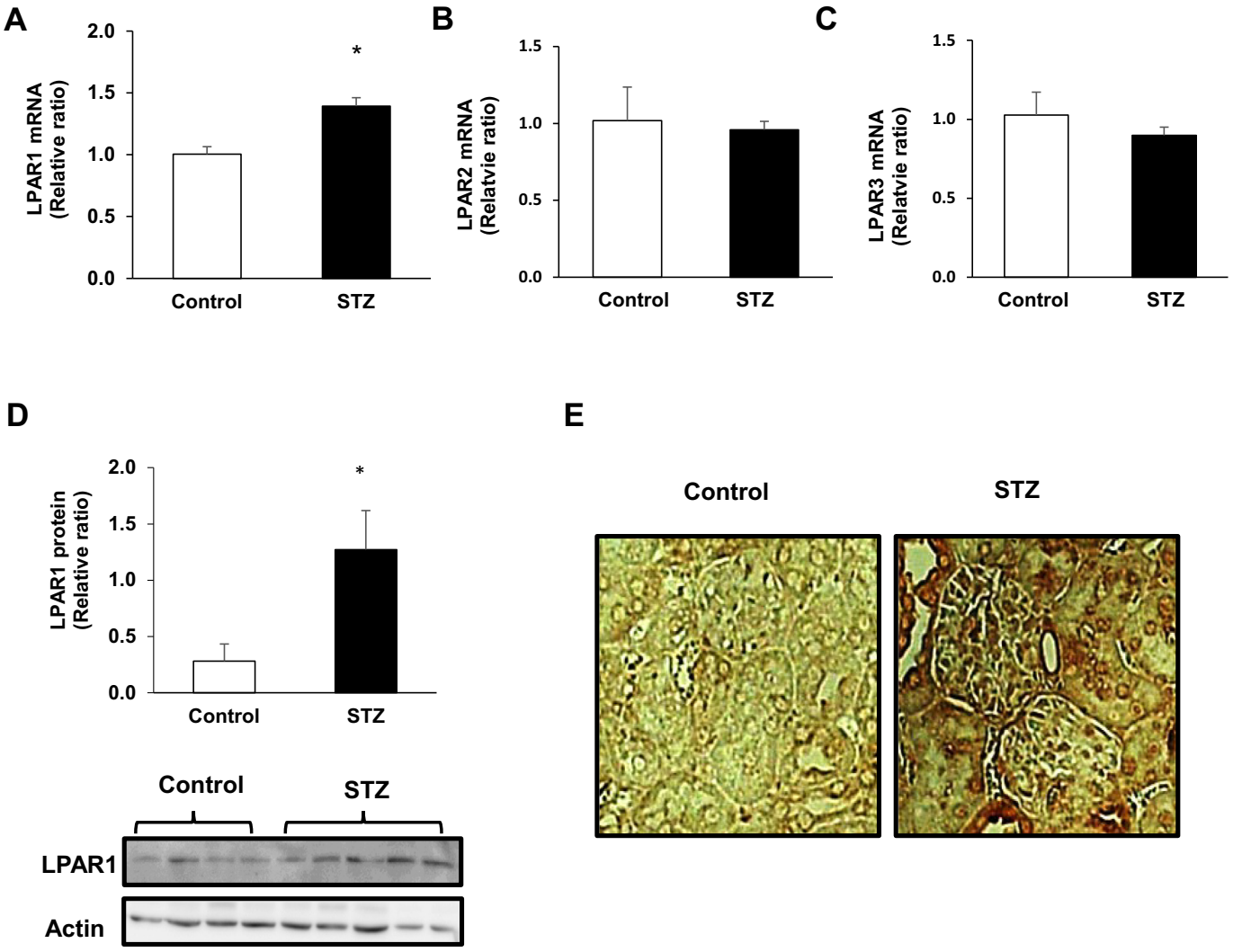

Fig. 2. The increase of LPAR1 expression is correlated with increase of oxidative stress in the kidney of STZ-induced diabetic mice. Total mRNA and proteins were extracted from kidney tissues of 16-week-old control and STZ-induced diabetic mice. (A–C) mRNA levels of (A) LPAR1, (B) LPAR2, and (C) LPAR3. (D) Protein levels of LPAR1 with a representative Western blot. (E) The kidney tissues were fixed in formalin, and then, subjected to immunohistochemical detection of 4-HNE (dark brown) as a marker for oxidative stress. *$p < 0.05$, ($n = 5$).

upregulated by STZ treatment, and these increases were reduced by 30 mg/kg of AM095 or losartan (Fig. 5E, F). Moreover, the protein expression levels of some key fibrotic mediators (COL1A and TIMP1) were significantly upregulated by STZ treatment and statistically reduced by AM095 treatment (Fig. 5G–I). In agreement with the fibronectin mRNA data, immunohistochemical staining for fibronectin in kidney tissue significantly increased in diabetic mice; however, it was reduced in AM095-treated diabetic mice (Supplementary Fig. 5).

4. Discussion

Recent studies have indicated that LPARs antagonism improves renal dysfunction in type 2 diabetic models [32,35]. Here, we demonstrated that a LPAR1-specific antagonist, AM095, attenuates diabetic nephropathy in a type 1 diabetic model through suppressing directly and/or indirectly the ROS-mediated inflammatory response. We further suggested that TLR4 and TLR4-dependent NADPH oxidase play a critical role in the pathogenesis of DN by downregulating NF-κB and JNK signaling.

Serum LPA and autotaxin levels are associated with proteinuria and kidney failure in the type 2 diabetic condition [27,36]. LPAR1-4 are expressed in the kidney tissue under normal conditions [37]. LPAR1 activation promotes tubulointerstitial fibrosis in mice given unilateral ureteral obstruction [26]. Our recent study and others have also demonstrated that LPARs-mediated signaling is activated in mesangial cells exposed to high glucose and in the renal cortex of a type 2 diabetic mouse model, whereas ki16425 or BMS002 treatment (antagonists for LPAR1/3) attenuated glomerular injury and recovered kidney dysfunction [32,35]. Consistent with these, our current study showed that LPAR1 expression is elevated in kidney of a STZ-induced diabetic mice. However, ki16425 and BMS002 have antagonistic effects for both LPAR1 and LPAR3 [32,35]. Thus, it is difficult to exclude the effects of antagonism on LPAR3 under pathogenic conditions, even though the expression of LPAR3 in renal cortex was not changed in the diabetic condition in our previous study [32]. In contrast, Zhang et al. showed that both LPAR1 and 3 are significantly increased in endothelial nitric oxide synthase-knockout ($eNOS^{-/-}$) *db/db* mice [35]. AM095 is well known as an antagonist for LPAR1 [30]. This characteristic of AM095 makes it more suitable for evaluating the role of LPAR1 on pathogenesis of DN, and thus, was used in the present study.

Our data showed that AM095 treatment significantly decreased blood glucose levels, although mildly, in two different cohorts of STZ-induced diabetic mice. This result is inconsistent with our previous study using ki16425 in the db/db mouse model, in which there was no

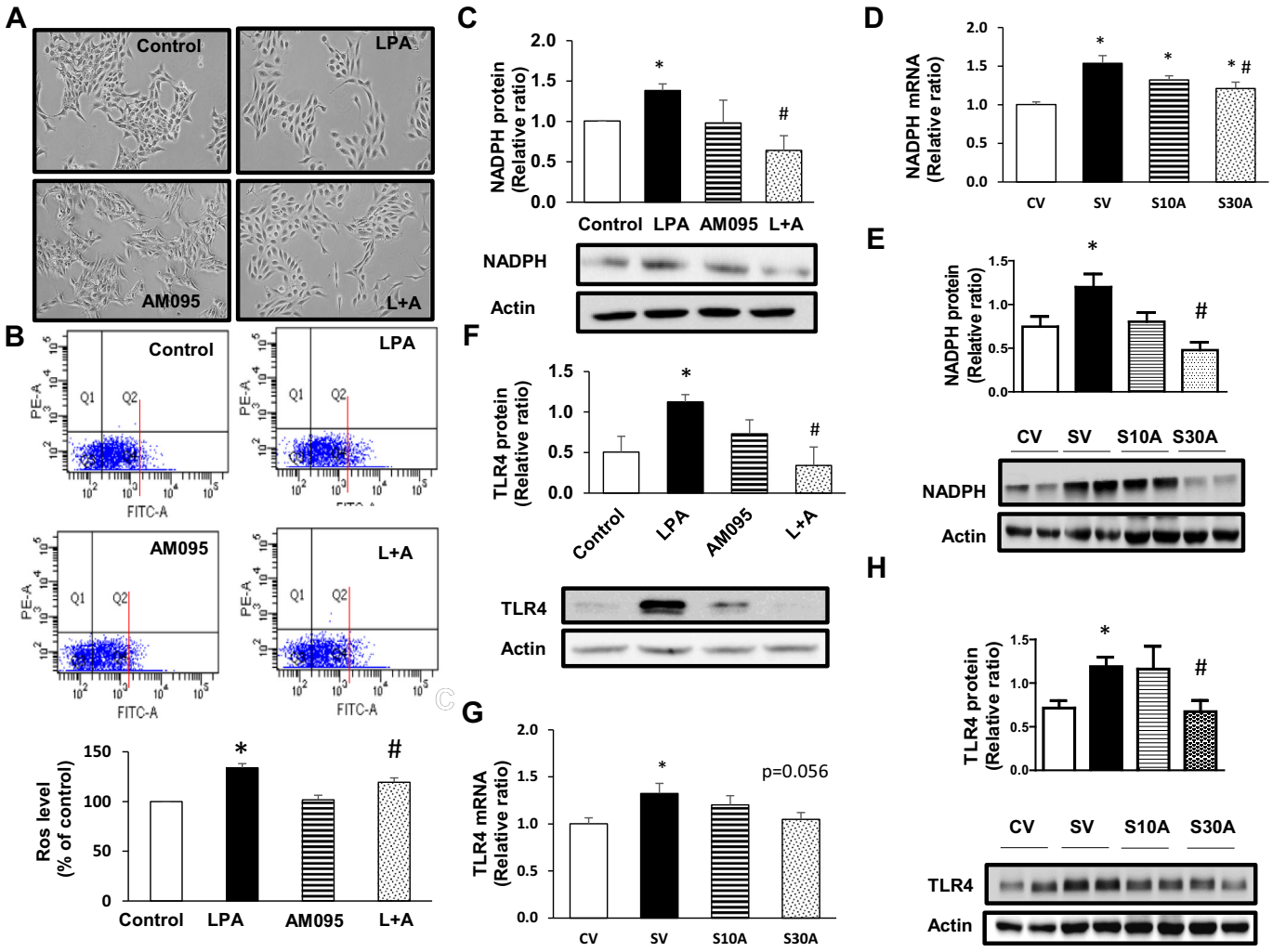

Fig. 3. AM095 treatment suppresses ROS production by down-regulating NADPH oxidase and TLR4 protein expression in mesangial cells treated with LPA and in diabetic mice. Mesangial cells were treated with or without AM095 with 5 μM of LPA for 3 h. ROS production levels were measured by flow cytometry by analyzing up to 1×10^4 cells. (A) Representative image of mesangial cells after treatment with either LPA, AM095, or both (L + A) (Original magnification, ×100). (B) ROS production levels with representative histogram of flow cytometry. (C) Protein levels of NADPH oxidase or (F) TLR4 with a representative Western blot. *$p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. LPA; n = 3–5. STZ-induced diabetic mice were treated with either vehicle (SV), or 10 (S10A) or 30 (S30A) mg of AM095. Mice not treated with STZ were used as a control (CV). (D) NADPH oxidase mRNA and (E) NADPH oxidase protein, (G) TLR4 mRNA and (H) TLR4 protein levels. *$p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. SV. (n = 6–8).

effect on blood glucose levels [32]. However, Rancoule et al. reported that LPAR antagonism using ki16425 significantly reduced blood glucose by increasing the number of islets and insulin secretion, as well as insulin sensitivity in liver and muscle in high fat diet-induced obese mice [27]. The discrepancy between these studies might be due to the different diabetic mouse models and the differences in the degree of affinity for LPAR1 between AM095 and ki16425.

Histological analysis of the kidney showed that AM095 administration reduced the impairment of kidney structure as shown by a smaller glomerular surface area and tuft area in the AM095 treatment group compared to the STZ-vehicle group. The therapeutic effect at 30 mg AM095 was also comparable with losartan treatment group. These results are consistent with our previous reports and others using a type 2 DN mouse model [32,35]. Taken together, the results indicated that AM095 administration may effectively improve kidney function by recovering the damaged kidney structure by reducing inflammatory mediated fibrosis.

Increasing evidence indicates that oxidative stress and ROS potentiate the development of DN [38]. Our data showed that the increase of LPAR1 expression in the kidney of STZ-induced diabetic mice was correlated with the increase of the detection of 4-HNE, a marker of oxidative stress. In addition, LPA increased ROS production in mesangial cells, and AM095 reduced this elevated ROS production. These data strongly suggested that AM095 treatment may inhibit renal damage by reducing ROS production.

Cellular ROS production is mediated by NADPH oxidase [39]. Our data showed that the expression of NADPH oxidase expression was increased by LPA treatment in SV40 MES13 cells and in the kidney of diabetic mice. This increase may, in turn, induce ROS production. TLR4 is an innate immune receptor classically activated by lipopolysaccharide in immune cells or by endogenous ligands in nonimmune cells [18,19]. TLR4 can activate the NF-κB pathway leading to inflammatory cytokines production. In diabetic patients, TLR4 expression and activation of its downstream signaling is observed in various cell types and organs [16,20]. As it is known that TLR4 also activates NADPH oxidase, leading to ROS production, we examined the changes of TLR4 expression in LPA-treated SV40 MES13 cells. We found that LPA treatment increased TLR4 expression in SV40 MES13 cells as well as in the kidney of diabetic mice. Consistent with our result, it has been previously reported that LPA increases TLR4 expression in THP-1 cells

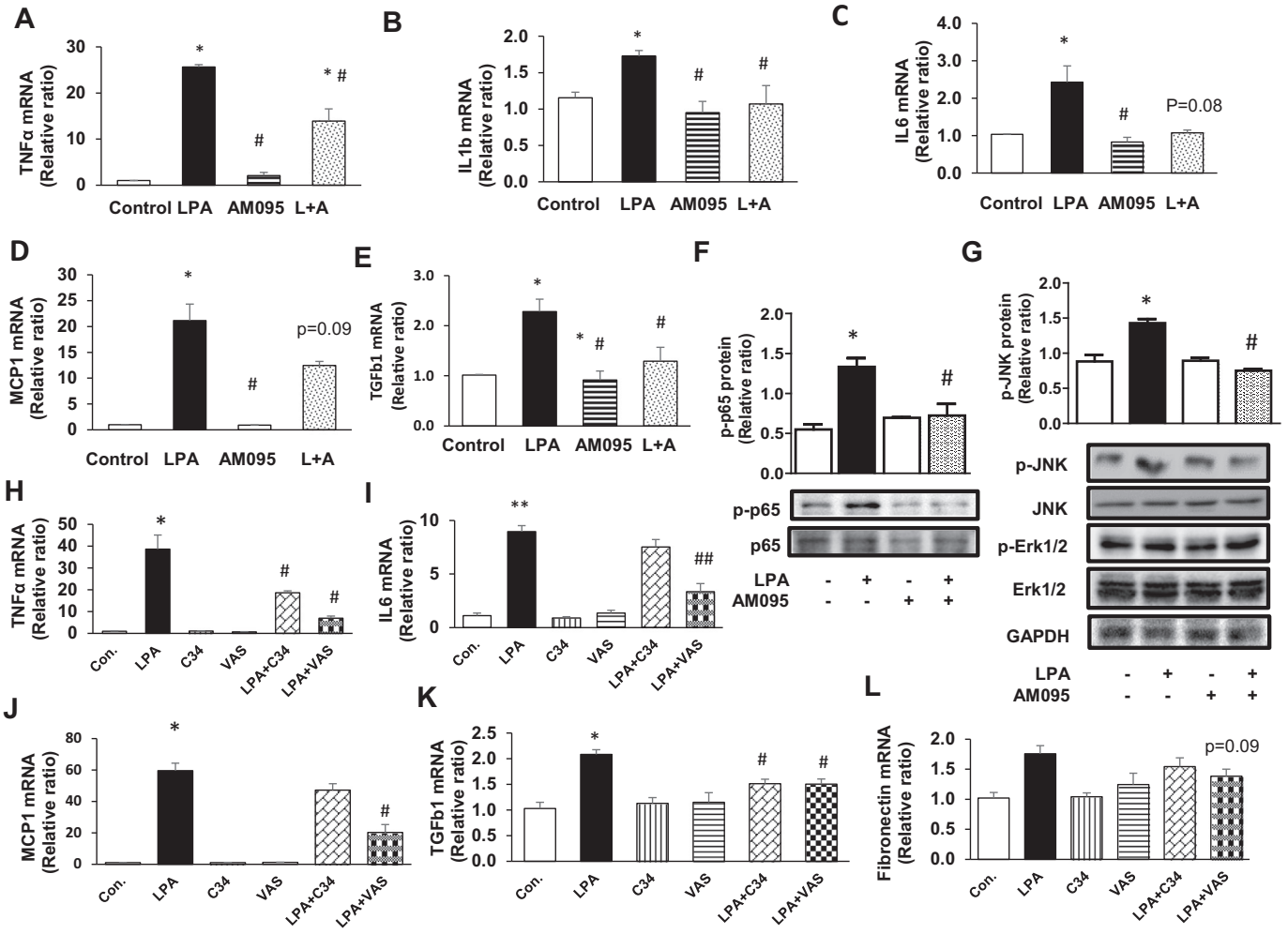

Fig. 4. AM095 treatment suppresses the expression of pro-inflammatory cytokines and fibrotic factors through NF-κB and JNK signaling pathways in mesangial cells treated with LPA. The cells were treated with 5 μM of LPA with or without AM095 (10 μM) for 3 h. (A) mRNA levels of TNFα, (B) IL1β, (C) IL6, (D) MCP1, and (E) TGFβ1 (n = 3–5). (F–G) Protein levels of p-NFκBp65 (p65), p-JNK, and p-ERK1/2 with representative Western blots. The cells were pre-treated with or without 10 μM of inhibitors targeting TLR4 (C34) or NADPH oxidase (VAS) 1 h before treatment with 5 μM of LPA for 3 h. mRNA levels of (H) TNFα, (I) IL6, (J) MCP1, (K) TGFβ1, and (L) fibronectin. * (n = 4–5). $p < 0.05$ vs. control, ** $p < 0.01$ VS. control, $^{\#}p < 0.05$ vs. LPA, $^{\#\#}p < 0.01$ vs. LPA.

[19]. Furthermore, our study showed that LPA induced TLR4 expression was blocked by LPA antagonism using AM095 in SV40 MES13 cells and in the kidney of diabetic mice, suggesting that the TLR4/NF-κB pathway plays an important role in the development of DN.

The activation of TLR4 by LPA may have dual functions. First, TLR4 can directly induce pro-inflammatory cytokines expression through phosphorylation of NFκBp65. Second, TLR4 can also activate NADPH oxidase, thereby increasing ROS production followed by elevated phosphorylation of NFκBp65 and JNK in LPA-treated mesangial cells. These two pathways may contribute to the induction of inflammatory molecules expression in the kidney. When we pretreated SV40 MES13 cells with pharmacological inhibitors for TLR4 or NADPH oxidase before challenging them with LPA, LPA-induced deleterious effects were reduced, as shown by the reduced pro-inflammatory cytokines and fibrotic factors expression. Moreover, TLR4 knockdown using siRNA technique also suppressed expressions of inflammatory cytokines and fibrotic factors. Collectively, these data suggested that the TLR4 and NADPH oxidase systems are important mediators of AM095 action to prevent the deleterious effects of LPA.

The expressions of IL6, TNFα, and IL1β, major pro-inflammatory cytokines secreted during tissue damage [40], are decreased in the kidney of AM095 treated mice compared with STZ-vehicle group. However, the expression of the inducer of microphage infiltration, MCP1, involved in early stage of inflammatory response was lowest expressed in STZ-vehicle group [41]. These results suggest that STZ-vehicle group may be further progressed in pathological stage compared with other treatment groups. The expression of fibrotic factors is regulated by inflammatory cytokines [42]. We found that the expression of TGFβ1 and fibronectin was higher in the diabetic condition and was lowered by AM095 treatment. Consistent with these data, the expressions of fibrotic factors, such as collagen and TIMP-1, were also reduced in the kidney of AM095-treated mice. Taken together, these data indicated that the decrease in inflammatory cytokine expression by AM095 treatment may contribute to the reduction of expressions of fibrotic factors.

5. Conclusion

Our study showed that blocking LPAR1 signaling by AM095 was highly effective in reducing the pathogenesis of DN by regulating TLR4 and NADPH oxidase directly associated with ROS production and pro-inflammatory signaling cascades activated by LPA in diabetic mice. LPAR1 antagonism might provide a potential target for therapeutic strategies to prevent or treat the development of DN.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2019.02.001.

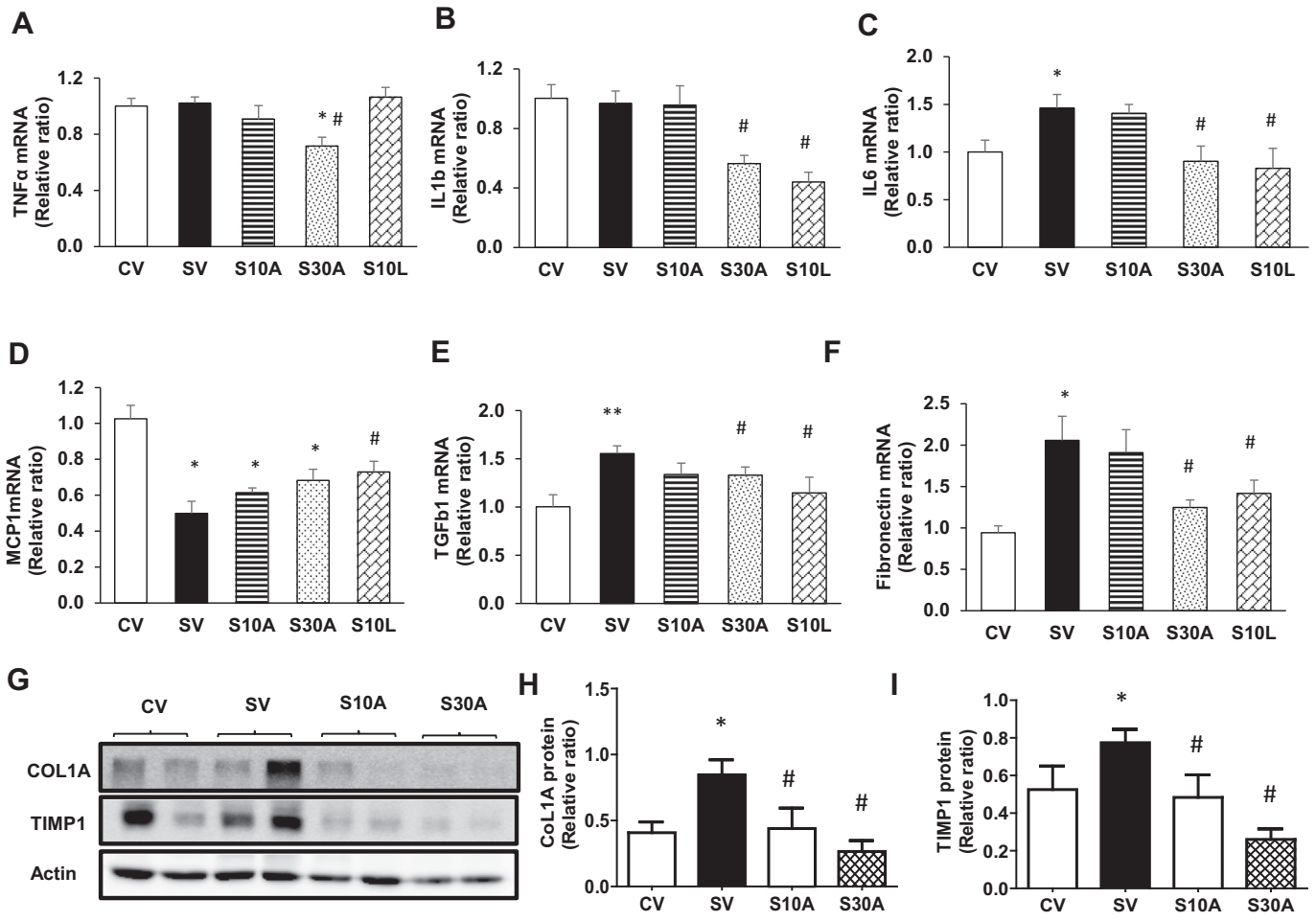

Fig. 5. AM095 treatment inhibits the expression of pro-inflammatory cytokines and fibrotic factors in the kidney of STZ-induced diabetic mice. STZ-induced diabetic mice were given either vehicle (SV), 10 mg (S10A) or 30 mg (S30A) of AM095, or 10 mg of losartan (S10L). Mice not treated with STZ were used as a control (CV). (A) mRNA levels of TNFα, (B) IL1β, (C) IL6, (D) MCP1, (E) TGFβ1, and (F) fibronectin. (G) A representative Western blot with quantification of protein levels of CoL1A1 (H) and TIMP1 (I). *$p < 0.05$ vs. control, **$p < 0.01$ vs. control, $^{\#}p < 0.05$ vs. SV. (n = 6–8).

Funding

This study was supported by grants from the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (NRF2016R1A2B2013347) and Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health and Welfare, Republic of Korea (grant number: HI14C1135).

Author contributions

J.H.L. designed and conducted the experiments, performed data analysis, and wrote the manuscript. M.K.S., H.C., and D.K performed experiments and data analysis. D.S. provided drugs. H.S.J. designed the study, interpreted the data, wrote the manuscript, and approved the final version of the manuscript for publication.

Conflict of interest

The authors declare that there is no duality of interest associated with this manuscript.

Transparency document

The Transparency document associated with this article can be found, in online version.

Acknowledgements

We thank Dr. Ann Kyle for editorial assistance.

References

[1] N. Bhattacharjee, S. Barma, N. Konwar, S. Dewanjee, P. Manna, Mechanistic insight of diabetic nephropathy and its pharmacotherapeutic targets: an update, Eur. J. Pharmacol. 791 (2016) 8–24.
[2] P. Fioretto, M. Mauer, Diabetic nephropathy: diabetic nephropathy-challenges in pathologic classification, Nat. Rev. Nephrol. 6 (2010) 508–510.
[3] H. Gallagher, R.J. Suckling, Diabetic nephropathy: where are we on the journey from pathophysiology to treatment? Diabetes Obes. Metab. 18 (2016) 641–647.
[4] K. Sharma, P. McCue, S.R. Dunn, Diabetic kidney disease in the db/db mouse, Am. J. Physiol. Ren. Physiol. 284 (2003) F1138–F1144.
[5] M.N. Budisavljevic, L. Hodge, K. Barber, J.R. Fulmer, R.A. Durazo-Arvizu, S.E. Self, M. Kuhlmann, J.R. Raymond, E.L. Greene, Oxidative stress in the pathogenesis of experimental mesangial proliferative glomerulonephritis, Am. J. Physiol. Ren. Physiol. 285 (2003) F1138–F1148.
[6] M. Brownlee, Biochemistry and molecular cell biology of diabetic complications, Nature 414 (2001) 813–820.
[7] S.B. Lee, R. Kalluri, Mechanistic connection between inflammation and fibrosis, Kidney Int. Suppl. (2010) S22–S26.
[8] C. Guijarro, J. Egido, Transcription factor-kappa B (NF-kappa B) and renal disease, Kidney Int. 59 (2001) 415–424.
[9] S. Segerer, P.J. Nelson, D. Schlondorff, Chemokines, chemokine receptors, and renal disease: from basic science to pathophysiologic and therapeutic studies, J. Am. Soc. Nephrol. 11 (2000) 152–176.
[10] W.A. Border, N.A. Noble, Transforming growth factor beta in tissue fibrosis, N.

Engl. J. Med. 331 (1994) 1286–1292.
[11] H.W. Schnaper, T. Hayashida, S.C. Hubchak, A.C. Poncelet, TGF-beta signal transduction and mesangial cell fibrogenesis, Am. J. Physiol. Ren. Physiol. 284 (2003) F243–F252.
[12] F.N. Ziyadeh, D.C. Han, J.A. Cohen, J. Guo, M.P. Cohen, Glycated albumin stimulates fibronectin gene expression in glomerular mesangial cells: involvement of the transforming growth factor-beta system, Kidney Int. 53 (1998) 631–638.
[13] X.M. Meng, D.J. Nikolic-Paterson, H.Y. Lan, TGF-beta: the master regulator of fibrosis, Nat. Rev. Nephrol. 12 (2016) 325–338.
[14] K. Vijay, Toll-like receptors in immunity and inflammatory diseases: past, present, and future, Int. Immunopharmacol. 59 (2018) 391–412.
[15] M.R. Dasu, S. Devaraj, L. Zhao, D.H. Hwang, I. Jialal, High glucose induces toll-like receptor expression in human monocytes: mechanism of activation, Diabetes 57 (2008) 3090–3098.
[16] M. Lin, W.H. Yiu, H.J. Wu, L.Y. Chan, J.C. Leung, W.S. Au, K.W. Chan, K.N. Lai, S.C. Tang, Toll-like receptor 4 promotes tubular inflammation in diabetic nephropathy, J. Am. Soc. Nephrol. 23 (2012) 86–102.
[17] T. Szasz, C.F. Wenceslau, B. Burgess, K.P. Nunes, R.C. Webb, Toll-like receptor 4 activation contributes to diabetic bladder dysfunction in a murine model of type 1 diabetes, Diabetes 65 (2016) 3754–3764.
[18] B. Beutler, Inferences, questions and possibilities in Toll-like receptor signalling, Nature 430 (2004) 257–263.
[19] B. Yang, Z. Zhou, X. Li, J. Niu, The effect of lysophosphatidic acid on Toll-like receptor 4 expression and the nuclear factor-kappaB signaling pathway in THP-1 cells, Mol. Cell. Biochem. 422 (2016) 41–49.
[20] M.R. Dasu, S. Devaraj, S. Park, I. Jialal, Increased toll-like receptor (TLR) activation and TLR ligands in recently diagnosed type 2 diabetic subjects, Diabetes Care 33 (2010) 861–868.
[21] F.Y. Ma, R.S. Flanc, G.H. Tesch, Y. Han, R.C. Atkins, B.L. Bennett, G.C. Friedman, J.H. Fan, D.J. Nikolic-Paterson, A pathogenic role for c-Jun amino-terminal kinase signaling in renal fibrosis and tubular cell apoptosis, J. Am. Soc. Nephrol. 18 (2007) 472–484.
[22] J.F. Navarro-Gonzalez, C. Mora-Fernandez, M. Muros de Fuentes, J. Garcia-Perez, Inflammatory molecules and pathways in the pathogenesis of diabetic nephropathy, Nat. Rev. Nephrol. 7 (2011) 327–340.
[23] M.J. Rane, Y. Song, S. Jin, M.T. Barati, R. Wu, H. Kausar, Y. Tan, Y. Wang, G. Zhou, J.B. Klein, X. Li, L. Cai, Interplay between Akt and p38 MAPK pathways in the regulation of renal tubular cell apoptosis associated with diabetic nephropathy, Am. J. Physiol. Ren. Physiol. 298 (2010) F49–F61.
[24] A.E. Kremer, J.J. Martens, W. Kulik, F. Rueff, E.M. Kuiper, H.R. van Buuren, K.J. van Erpecum, J. Kondrackiene, J. Prieto, C. Rust, V.L. Geenes, C. Williamson, W.H. Moolenaar, U. Beuers, R.P. Oude Elferink, Lysophosphatidic acid is a potential mediator of cholestatic pruritus, Gastroenterology 139 (2010) 1008–1018 (1018 e1001).
[25] J. Pfeilschifter, C. Schalkwijk, V.A. Briner, H. van den Bosch, Cytokine-stimulated secretion of group II phospholipase A2 by rat mesangial cells. Its contribution to arachidonic acid release and prostaglandin synthesis by cultured rat glomerular cells, J. Clin. Invest. 92 (1993) 2516–2523.
[26] J.P. Pradere, J. Klein, S. Gres, C. Guigne, E. Neau, P. Valet, D. Calise, J. Chun, J.L. Bascands, J.S. Saulnier-Blache, J.P. Schanstra, LPA1 receptor activation promotes renal interstitial fibrosis, J. Am. Soc. Nephrol. 18 (2007) 3110–3118.
[27] C. Rancoule, C. Attane, S. Gres, A. Fournel, R. Dusaulcy, C. Bertrand, C. Vinel, K. Treguer, M. Prentki, P. Valet, J.S. Saulnier-Blache, Lysophosphatidic acid impairs glucose homeostasis and inhibits insulin secretion in high-fat diet obese mice, Diabetologia 56 (2013) 1394–1402.
[28] K.J. Grove, P.A. Voziyan, J.M. Spraggins, S. Wang, P. Paueksakon, R.C. Harris, B.G. Hudson, R.M. Caprioli, Diabetic nephropathy induces alterations in the glomerular and tubule lipid profiles, J. Lipid Res. 55 (2014) 1375–1385.
[29] F.V. Castelino, J. Seiders, G. Bain, S.F. Brooks, C.D. King, J.S. Swaney, D.S. Lorrain, J. Chun, A.D. Luster, A.M. Tager, Amelioration of dermal fibrosis by genetic deletion or pharmacologic antagonism of lysophosphatidic acid receptor 1 in a mouse model of scleroderma, Arthritis Rheum. 63 (2011) 1405–1415.
[30] J.S. Swaney, C. Chapman, L.D. Correa, K.J. Stebbins, A.R. Broadhead, G. Bain, A.M. Santini, J. Darlington, C.D. King, C.S. Baccei, C. Lee, T.A. Parr, J.R. Roppe, T.J. Seiders, J. Ziff, P. Prasit, J.H. Hutchinson, J.F. Evans, D.S. Lorrain, Pharmacokinetic and pharmacodynamic characterization of an oral lysophosphatidic acid type 1 receptor-selective antagonist, J. Pharmacol. Exp. Ther. 336 (2011) 693–700.
[31] H.R. Jin, W.J. Kim, J.S. Song, S. Piao, M.J. Choi, M. Tumurbaatar, S.H. Shin, G.N. Yin, G.Y. Koh, J.K. Ryu, J.K. Suh, Intracavernous delivery of a designed angiopoietin-1 variant rescues erectile function by enhancing endothelial regeneration in the streptozotocin-induced diabetic mouse, Diabetes 60 (2011) 969–980.
[32] H.Y. Li, Y.S. Oh, J.W. Choi, J.Y. Jung, H.S. Jun, Blocking lysophosphatidic acid receptor 1 signaling inhibits diabetic nephropathy in db/db mice, Kidney Int. 91 (2017) 1362–1373.
[33] A.R. Pradeep, E. Agarwal, P. Bajaj, N.S. Rao, 4-Hydroxy-2-nonenal, an oxidative stress marker in crevicular fluid and serum in type 2 diabetes with chronic periodontitis, Contemp. Clin. Dent. 4 (2013) 281–285.
[34] W.J. Zhang, H. Wei, B. Frei, Genetic deficiency of NADPH oxidase does not diminish, but rather enhances, LPS-induced acute inflammatory responses in vivo, Free Radic. Biol. Med. 46 (2009) 791–798.
[35] M.Z. Zhang, X. Wang, H. Yang, A.B. Fogo, B.J. Murphy, R. Kaltenbach, P. Cheng, B. Zinker, R.C. Harris, Lysophosphatidic acid receptor antagonism protects against diabetic nephropathy in a type 2 diabetic model, J. Am. Soc. Nephrol. 28 (2017) 3300–3311.
[36] G. Ferry, E. Tellier, A. Try, S. Gres, I. Naime, M.F. Simon, M. Rodriguez, J. Boucher, I. Tack, S. Gesta, P. Chomarat, M. Dieu, M. Raes, J.P. Galizzi, P. Valet, J.A. Boutin, J.S. Saulnier-Blache, Autotaxin is released from adipocytes, catalyzes lysophosphatidic acid synthesis, and activates preadipocyte proliferation. Up-regulated expression with adipocyte differentiation and obesity, J. Biol. Chem. 278 (2003) 18162–18169.
[37] S. Yokoyama, Self-associated tetramer of human apolipoprotein E does not lead to its accumulation on a lipid particle, Biochim. Biophys. Acta 1047 (1990) 99–101.
[38] N. Kashihara, Y. Haruna, V. K. Kondeti, Y. S. Kanwar, Oxidative stress in diabetic nephropathy, Curr. Med. Chem. 17 (2010) 4256–4269.
[39] M. Sedeek, R. Nasrallah, R.M. Touyz, R.L. Hebert, NADPH oxidases, reactive oxygen species, and the kidney: friend and foe, J. Am. Soc. Nephrol. 24 (2013) 1512–1518.
[40] M.D. Turner, B. Nedjai, T. Hurst, D.J. Pennington, Cytokines and chemokines: at the crossroads of cell signalling and inflammatory disease, Biochim. Biophys. Acta 1843 (2014) 2563–2582.
[41] C. Shi, E.G. Pamer, Monocyte recruitment during infection and inflammation, Nat. Rev. Immunol. 11 (2011) 762–774.
[42] L.A. Borthwick, T.A. Wynn, A.J. Fisher, Cytokine mediated tissue fibrosis, Biochim. Biophys. Acta 1832 (2013) 1049–1060.