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Cross-talk between angiotensin-II and toll-like receptor 4 triggers a synergetic inflammatory response in rat mesangial cells under high glucose conditions

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ABSTRACT

Toll-like Receptor 4 (TLR4) may play an important role in the pathogenesis of diabetic nephropathy (DN). In this study, We observed the TLR4 signal and the release of inflammation factors after angiotensin II (Ang II) stimulation in rat mesangial cells (MCs) under high glucose conditions, this revealed the innate immune mechanism of injury by Ang II in DN. Our data showed that TLR4 and MyD88 were up-regulated significantly in high glucose and AngII-induced MCs; meanwhile, NF-κB as well as MCP-1, IL-6 were also highly expressed. In cells that were transfected with TLR4 SiRNA , the parameters were greatly inhibited; similar effects were detected in cells that were treated with Irbesartan. We concluded that Ang II synergized with high glucose in the release of pro-inflammatory factors mainly through the upregulation of TLR4 signaling in MCs, Cross-talk between Ang II and TLR4 contributed to the MC inflammatory injury under high glucose conditions.

1. Introduction

Diabetic nephropathy (DN) is the leading cause of end stage renal disease (ESRD). Clinical trials found that numerous DN patients progress to ESRD despite improved glycemic control and the renin angiotensin aldosterone system(RAAS) blockade [1,2]. New evidence suggests that inflammation is implicated in the degeneration of renal function [3–5]. Renal inflammatory and oxidative stress may result from the metabolic and hemodynamic derangements observed in DN [5,6], however, the specific mechanism of the inflammatory response is still not clear. Revealing a novel mechanism could provide a new strategy for arresting the progression of renal disease.

The latest studies have focused on the innate immune mechanism that is concerned with the inflammation in diabetes [7,8]. Toll-like receptors(TLRs) are a conserved family of pattern recognition receptors that have been shown to be mediated by innate immune and inflammatory responses [9,10]; TLR4 was the first identified molecule of TLRs that was activated by combined with the corresponding ligand, and was involved in many kidney diseases [11,12]. The expression of TLR4 in diabetes was increasing [13,14], and several studies have documented that high glucose levels upregulates TLR4 expression in renal mesangial(MC) and tubular epithelial cells [15,16], This suggests that TLR4 may have a prominent role in the activation of the innate immune response and the renal local stress in DN [15].

Glomerular mesangium metabolic abnormalities under high glucose conditions is one of the main characteristics of DN [17,18]. Evidences suggested that the intra-renal RAAS especially Angiotensin II(Ang II) leads to MC metabolic abnormalities and plays a “danger” factor in DN [19–21]. Previously, our group demonstrated that Ang II upregulated TLR4 expression, resulting in MC oxidative stress and apoptosis [22]. The latter study and others have also shown that blockade of the RAAS could attenuate TLR4 expression and the downstream pro-inflammatory and apoptotic events [23,24], suggesting that intrarenal RAAS activation might be another mechanism of renal TLR4 activation in DN. Although various detrimental effects of Ang II have been suggested [25–28], its exact mechanism of action as a non-hemodynamic danger factor

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and its relation to renal innate immune receptor-TLR4 in DN are still not well understood. It is important to clarify the interactions among Ang II, TLR4, and inflammation in MCs under hyperglycemia; this may have clear implications for DN via the innate immune system. Therefore, in this study, we mimicked a state of RAAS activation by adding a high concentration of Ang II to rat MCs under high glucose conditions, we subsequently evaluated the expressions of TLR4 signaling as well as inflammatory cytokines in MCs; By siRNA-mediated knockdown of TLR4, we further investigated the cross-talk between Ang II and TLR4 signaling under hyperglycemia conditions in MCs.

2. Materials and methods

2.1. Reagents

Rat glomerular Mesangial cell lines(HBZY-1) were obtained from China Center for type culture collection (Wuhan, China). RNA extraction kit and Ang II (sigma, USA), reverse transcription reagents and PCR primers PCR reagent synthesis and TLR4 SiRNA (Invitrogen, CA, USA), DMEM and fetal bovine serum were purchased from Hyclone (USA). IL-6 and MCP-1 ELISA kits were purchased from shanghai (Shanghai Westdon, China). Antimouse TLR4 antibodies and antibody of MyD88, NF-κB (Abcam, Cambridge, UK). Irbsartan(Angiotension receptor bloker, Sanofi).

2.2. Cell culture

HBZY-1 were cultured in growth media comprised of endotoxin free DMEM media supplemented with 5.6 mM glucose, 10% FBS, 100 U/ml penicillin and 100 ug/ml streptomycin in a humidified atmosphere of 5% CO2 and 95% air at 37 °C until they exhibited a typical hill and valley growth pattern. The cells(HBZY-1) were digested by 0.25% of trypsin at 2 min, if the cells were observed scattered, then termination of digestion. The media was replaced every 72 h. MC grown to passage 5 were used for all experiments.

3. Treatments

When the cells were grown to 80–90% confluence in six-well plates, the medium was changed to serum free medium for an additional 24 h culture before any further treatments. The cells were exposed to 5.5 mM glucose(NG), 25 mM glucose(HG), and 19.5 mM mannitol was added along with 5.5 mM glucose as an osmotic control for 12 and 24 h. Cells incubated with Ang II (10^{-7} M) and 5.5 mM glucose for 12 h or 24 h were used as the stimulation group. In order to exclude the effect of Angiotension receptor(AT1R), equal number of cells were added with 10^{-5} M Irbesartan (AT1R Blocker, ARB) for 1 h and then incubated with 25 mM glucose for 12 h or 24 h in another group. Immunofluorescence was used to observe TLR4 protein expression. Cell supernatants, lysates, and RNA were collected and used for ELISA, Western blotting, and real-time PCR, respectively.

3.1. Immunofluorescence staining

TLR4 surface expression was determined by immunofluorescence staining. The preparation of cells to climb, according to the predetermined requirement processing cell, PBS as a negative control. HBZY-1 grown on coverslips were fixed with 4% paraformaldehyde, blocked with 10% BSA and stained with anti-TLR4 antibody, followed by FITC-conjugated IgG, and then mounted with Vectashield containing 6-diamidino-2-phenylindole. Immunofluorescence was visualized under a Leica fluorescence microscope with the appropriate filters.

3.2. RNA extraction and real-time PCR

Total RNA was isolated from cells using Omega EANA™. Total RNA kitII(Omega, USA). cDNA was synthesized from 1 μg of total RNA by using TransScript First-Strand cDNA Synthesis SuperMix Kit(Invitrogen, USA) and following the manufacturer's instructions. Gene expression was analyzed by real-time PCR in an ABI Prism 7500 sequence detection system. The sample were run in triplicate. Primers for rat TLR4, MyD88 and β-actin purchased from Invivogen following manufacturers cycling parameters. TLR4: forward 5′-TCG GTG GTC AGT GTG CTT GTG G −3′, reverse 5′-AAA GCT GAA AGC GGG GCA CTC C-3′,MyD88: forward 5′-TCA ACA AGC GAG CGC ACC GT -3′, reverse 5′-TGA GCG CGA CCA ACG GTA GA-3′,β-actin: forward 5′-TCA GGT CAT CAC TAT CGG CAA T −3′, reverse 5′-AAA GAA AGG GTG TAA AAC GCA -3′. Amplification of β-actin was used as loading control. The relative quantities of amplified cDNAs were analyzed by SDS software (Applied Biosystems) and target values were normalized to β-actin mRNA.

3.3. Transient transfection with siRNAs

The small interfering RNA (siRNA) duplexes corresponding to TLR4 and normal control SiRNA were from Invitrogen. Transient transfection of siRNA was carried out using Lipofectamine 2000 transfection reagent (Invitrogen, CA). SiRNA (75 nM) was formulated with Lipofectamine 2000 transfection reagent according to the manufacturer's instruction.

3.4. Western blot analysis

Western blot was performed to determine the expression of TLR4 and MyD88 protein as described previously. Briefly, protein were extracted by 160 μl of RIPA lyse buffer, and concentration was determined with the BCA protein assay kit. 20 μg of total protein was resolved under denaturing conditions in an 8% SDS-PAGE gel and eletroblotted onto a nitrocellulose membrane. And probed with antibodies for TLR4, MyD88, nuclear factor-κB (NF-κB), followed by washing and incubation with HRP conjugated secondary antibodies, the detection was performed with the ECL (Thermo Fisher Scientific) according to the manufacturers'instructions, and the stripped membranes were further probed with β-actin as loading control. The intensity of the bands was analyzed with Image J software. Data is presented as fold induction normalized to β-actin. Results were expressed as relative to control. Each experiment was repeated three times.

3.5. Enzyme linked immunosorbent assay (ELISA)

Collecting cell supernatant of each group on 24 h, then the expression of Interleukin(IL)-6 and Monocyte chemotactic protein-1(MCP-1) were measured by ELISA Kit according to the manufacturers'instructions. Inflammatory cytokines were measured in the supernatant at 24 h of various treatments, Each group of experiment repeated three times. OD value of the sample was determined at 450 nm wavelength by enzyme standard instrument. The calculation of the corresponding concentration of each sample according to the standard curve and the OD value.

3.6. Statistical analysis

Statistical analyses were performed using spss18.0 software. All data were expressed as mean ± standard deviation of at least three representative independent experiments. Differences were analyzed by oneway ANOVA and probability value of $P < 0.05$ was considered significant.

4. Results

1. Immunofluorescence staining results of TLR4 surface expression

Immunofluorescence staining were detected the expression of TLR4 protein of MCs stimulated by HG and/or Ang II after 24 h. Mean fluorescence intensity of TLR4 expression significantly increased in the presence of HG or Ang II compared with NG or the mannitol control ($P < 0.01$). In the HG + Ang II group, TLR4 expression was greatly increased, Irbesartan and TLR4 SiRNA significantly down-regulated TLR4 protein expression significantly ($P < 0.01$; Fig. 1).

2. Effect of high glucose or Ang II on TLR4 and MyD88 mRNA expression in MCs

MCs were exposed to 5.5 mM glucose(NG) and 25 mM glucose(HG) for 12 h, at the same time, as an osmotic control, cells were exposed to 19.5 mM mannitol added to 5.5 mM glucose. The effects of 25 mM glucose or 10^{-7} M Ang II on the mRNA expression of TLR4 and MyD88 were analyzed by real-time PCR. The addition of mannitol did not result in a significant increase in TLR4 expression, thus excluding the possibility that a glucose-induced increase in TLR4 mRNA expression was an osmotic effect. As shown in Fig. 2, the expression of TLR4 and MyD88 mRNA in cells exposed to 25 mM glucose or 10^{-7} M Ang II co-incubated with 5.5 mM glucose, both increased markedly when compared to NG group ($P < 0.01$) ; For cells that were co-stimulated with high glucose and AngII, the expression of MyD88 mRNA was significantly further increased compared with the HG or AngIItreated groups (both $P < 0.01$). Irbesartan down-regulated TLR4 signaling that was stimulated by high glucose ($P < 0.01$ compared to HG group). TLR4 SiRNA further down-regulated the expression of TLR4/MyD88 mRNA (both $P < 0.01$).

3. Western-blot of TLR4/MyD88 and NF-κB proteins stimulated by Ang II or high glucose in MCs

The effect of HG or Ang II on the expression of TLR4 and MyD88 proteins was further analyzed by Western blotting. As shown in

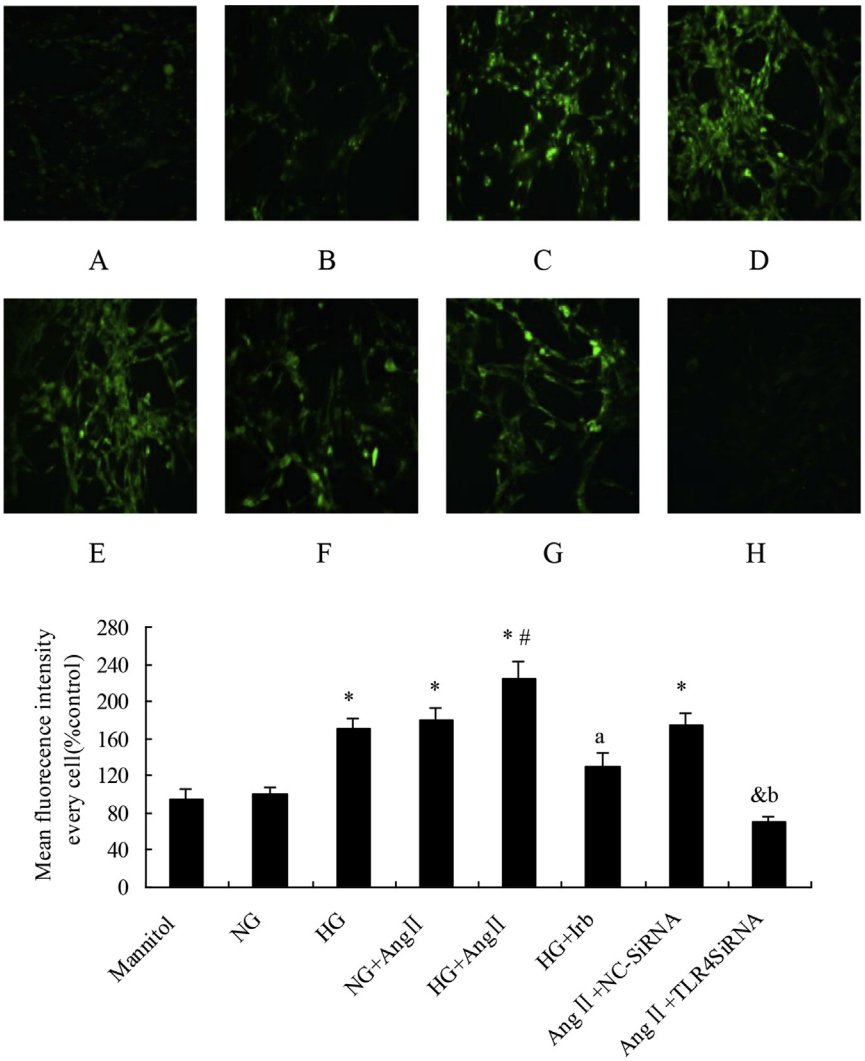

Fig. 1. TLR4 protein expression in cell surface of HBZY-1 detected by immunofluorescence staining. (A)Mannitol; (B) cells cultured with 5.5 mmol/L glucose media, NG; (C) cells cultured with 25 mmol/L glucose media, HG (D) 10^{-7} M Angiotension IIwas added to 5.5 mM glucose, NG + Ang II; (E) 10^{-7} M Angiotension IIwas added to 25 mM glucose, HG + Ang II; (F) cells pre-treated with 10^{-5} M Irbesartan for 1 h and then with 25 mM glucose, HG + Irb. (G) 10^{-7} M Angiotension IIwith Negtive Control SiRNA, Ang II + NC-SiRNA;(H) 10^{-7} M Angiotesion II with TLR4 SiRNA, Ang II + TLR4 SiRNA ($^{*}p < 0.01$, compared with NG or Mannitol, $^{\#}p < 0.01$, compared with HG or Ang II group,$^{a}P < 0.05$, comared with HG, $^{\&}p < 0.01$, compared with Ang II + NC-SiRNA, $^{b}p < 0.05$ compared with HG + Irb).

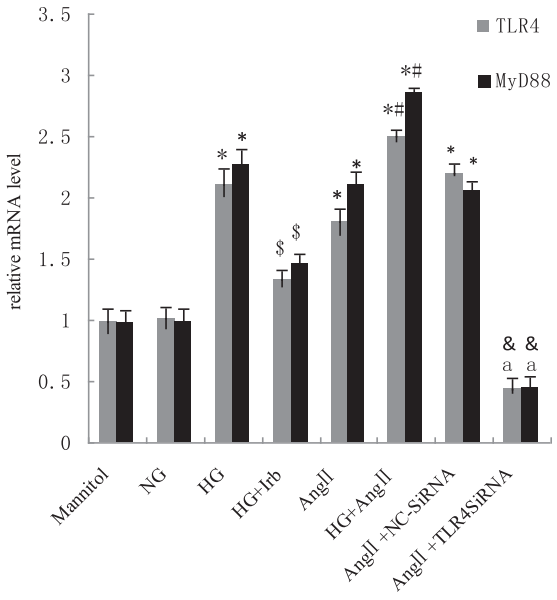

Fig. 2. Effect of High glucose or AngIIon TLR4/MyD88 mRNA expression in HBZY-1. mRNA expression was determined by real-time PCR. All results represent means ± SD obtained from three independent experiments. NG: cells cultured with 5.5 mmol/L glucose media. Mannitol : 19.5 mM mannitol was added to 5.5 mM glucose as osmotic control. HG: cells cultured with 25 mmol/L glucose media. AngII: Angiotension II, 10^{-7} M. Irb: Irbesartan, 10^{-5} M. NC-SiRNA : Negtive Control SiRNA ($^{*}p < 0.01$, compared with NG or Mannitol, $^{\#}P < 0.01$, comared with HG or Ang II. $^{\$}p < 0.05$, comared with HG, $^{\&}p < 0.01$, compared with Ang II + NC-SiRNA, $^{a}p < 0.01$ compared with HG + Irb).

Fig. 3A/B, compared to the NG group, the expression of TLR4 and MyD88 proteins in MCs that were exposed to HG and 10^{-7} M Ang II, increasesd greatly after 24 h. For cells that were co-stimulated with HG and Ang II, the expression of TLR4 and MyD88 protein was significantly further increased (both $P < 0.01$) compared to the HG-only group. Irbesartan can down-regulate TLR4 signaling that is stimulated by HG. TLR4 SiRNA further down regulated the expression of TLR4/MyD88 proteins ($P < 0.01$) . The expression of NF-kB p65 protein was also detected by western blotting, as shown in Fig. 3. Compared to NG, NF-kB protein expression in the HG and Ang II group increased significantly; Compared to the HG or Ang II groups, NF-kB protein expression showed a significant further increase in the group co-stimulated with HG and Ang II ($P < 0.01$).

4. Up-regulation of IL-6 and MCP-1 proteins by Ang II or HG in MCs

Because activation of NF-κB p65 by TLR4 leads to activation of various proinflammatory genes, we compared the levels of inflammatory cytokines such as IL-6, MCP-1 and TNF-α in the supernatants at 24 h. As shown in Fig. 4, the expression of MCP-1 and IL-6 but not TNF-α in cells exposed to HG or Ang II, increased greatly after 24 h, compared with the NG group. In cells that were co-stimulated with HG and Ang II, the expression levels of MCP-1 and IL-6 protein were significantly increased still further ($P < 0.01$). Compared to the HG group, Irbesartan was able to down-regulate MCP-1 and IL-6 protein in cells that were stimulated with Ang II. Compared to the HG or HG with Irb groups, TLR4 SiRNA further and significantly down-regulated the expression of MCP-1 and IL-6 protein (both $P < 0.01$).

5. Discussion

In recent years, the role of innate immune receptor–TLRs in renal diseases has been increasingly recognized [11,12]. Various studies have provided evidence that link TLR4 and renal cell injury in DN [13,14], the details are still under active consideration [15,16]. TLR4 can facilitate harmful responses through a signaling cascade that involves myeloid differentiation factor 88 (MyD88), interleukin-1 receptor-associated kinase (IRAK), etc, which may lead to the activation of transcriptional factor NF-κB and subsequent regulation of inflammatory factors [10]. High glucose has been considered as the initiating factor in various pathologies of diabetes; and MCs are more prone to oxidative stress and

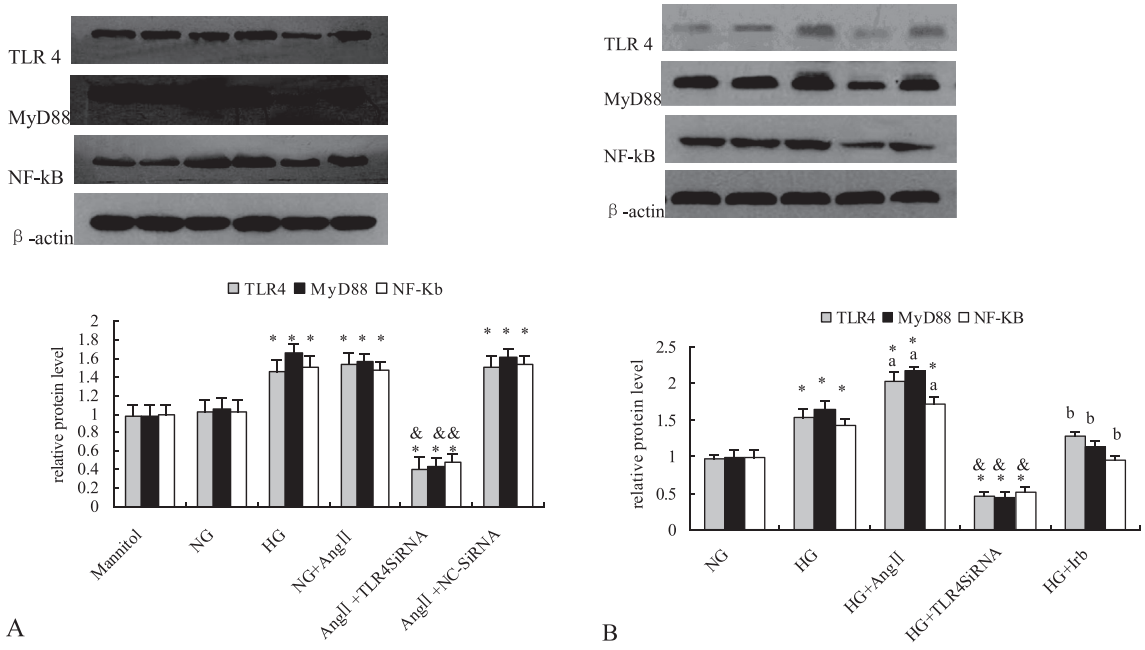

Fig. 3. Detection of TLR4/MyD88 and NF-κB protein expression using Western blots incubated with specific antibodies. This blot is representative of three independent experiments with qualitatively similar changes. (A: $^{*}p < 0.01$,compared with NG or Mannitol; $^{\&}p < 0.01$, Comared with NG + Ang II or Ang II + NC-SiRNA. B: $^{*}p < 0.01$ compared with NG; $^{\&}P < 0.01$, compared with HG + Ang II; $^{a}P < 0.01$, comared with HG ; $^{b}p < 0.05$, compared with HG).

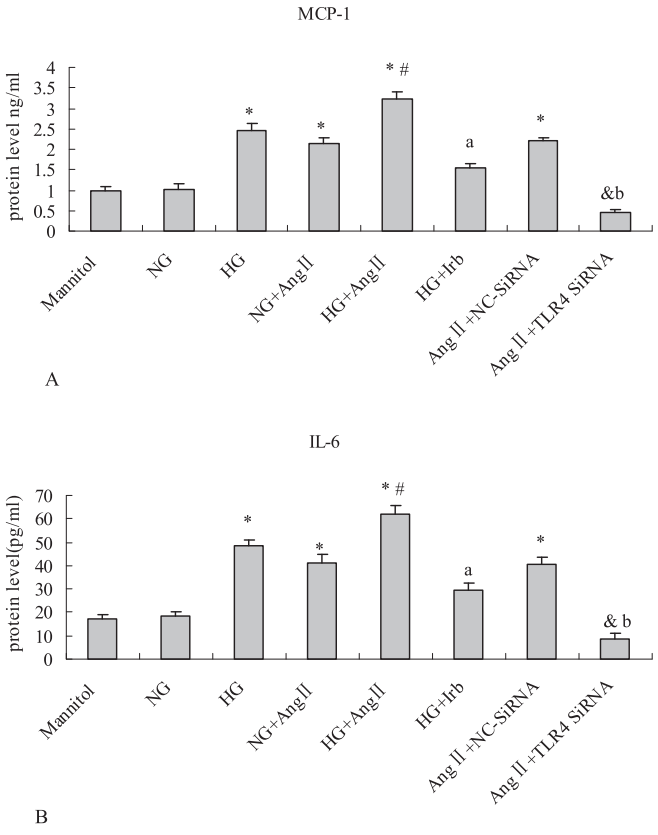

Fig. 4. Levels of cell supernatant inflammatory factors MCP-1(A)and IL-6(B). ($^{*}p < 0.01$, compared with NG or Mannitol; $^{\#}p < 0.01$, compared with HG or Ang II, $^{a}P < 0.05$, comared with HG, $^{\&}p < 0.01$, compared with Ang II + NC-SiRNA, $^{b}p < 0.01$ compared with HG + Irb).

inflammation under high glucose conditions [22]. Although inflammation is traditionally thought of as the result of macrophage infiltration, the present study demonstrated that there is significantly increased TLR4 as well as its downstream signal MyD88 expression in glomerular MCs under 25 mM high glucose condition; Furthermore, there was a significant increase in NF-kB p65 expression and the secretion of inflammatory biomediators (IL-6 and MCP-1). The current innate immune studies have lead to the novel idea that renal inherent cells, such as MCs and TECs [24], can produce cytokines and can express molecules that are part of the local chronic inflammatory response.

Interestingly, our study firstly found that Irbesartan(AT1 receptor bloker, ARB) directly downregulated the expression of the TLR4 signal as well as inflammatory molecules that were activated by high glucose levels (25 mM). In addition, we observed that HG increased MC intracellular reactive oxygen species (ROS) production, both TLR4 SiRNA and Irbesartan can modulate the accumulation of ROS (data not shown). Our previous study revealed the novel mechanism by which ARB helps to normalize ROS production by modulation of the TLR4/MyD88 pathway in MCs and produces an antioxidant-like effect [22]. Also, Kaur et al.[16] reported that high glucose induces the expression of ROS in an *in vitro* normal human MC model. And hyperglycemia-induced TLR4 expression is associated with increased NADPH oxidase activity via PKC-δwhich plays a crucial role in the generation of ROS [29]. These findings help us understand deeply the intrinsic link between innate immune system and oxidative stress of MCs in DN.

Ang II can lead to glomerular mesangial cell synthetic metabolic abnormalities [20], considerable evidence suggests that the intrarenal RAAS especially Ang II plays an important role in DN [19–21]. Although various detrimental effects of Ang II have been suggested, the molecular details are not completely understood so far. The classic view from previous studies claimes that Ang II plays a role in renal inflammation mainly through AT1R [30]. However, our study found that ARBs have the potential to reduce renal stress through inhibition of TLR4 expression under high glucose conditions; TLR4 SiRNA could down regulate the expression of TLR4 and attenuated the expression of proinflammatory factors in MCs stimulated by Ang II; Our previous study also suggested that Ang II can serve as a "danger" factor by modifying TLR4 expression [22], moreover, the expression of TLR4 and inflammation factors can be inhibited by AT1R blocker both *in vivo* and *in vitro* [22,24], which strongly suggests that the Ang II-induced MC injury involves a novel innate immune-related mechanism. Wolf et al. have been reported that the activating protein-1 (AP-1) and an Et-26 specific sequence (Ets) binding site in the TLR4 promoter are responsible for the TLR4 gene activity in MCs stimulated by Ang II [27]. The present study demonstrated that Ang II induced TLR4 and MyD88 activity in rat MCs, NF-κB protein also increased significantly, resulting in the chemokine and inflammation factors such as MCP-1 and IL-6 being markedly released. This suggested the possibility that an AngII-mediated up-regulation of TLR4 could also be relevant for the development of MC inflammation in DN.

In order to elucidate the synergetic effects of Ang II under high glucose, we further observed the changes of TLR4 signaling by HG and Ang II joint intervention. Immunoflorescence and Western blot as well as ELISA data demonstrated that in MCs co-stimulated with HG and Ang II, TLR4/MyD88/NF-κB protein and the inflammation factors showed further remarkable increases compared with the individual HG or Ang II groups; On the other hand, SiRNA-mediated knockdown of TLR4 results a markedly attenuation of MyD88/NF-κB proteins and the inflammation factors expression; Irbesartan(ARB), directly downregulated the expression of TLR4 and its adaptors as well as inflammatory molecules which were activated by HG, strongly suggested that this capacity of Irbesartan is depends on TLR4 activation rather than AT1. The NF-kB dependent chemokine and cytokine MCP-1 and IL-6, have been implicated in the progression of DN as demonstrated by our group and others [24,31,32], Based on the above findings, we predicted that Ang II may act synergistically with HG to promote a pro-inflammatory response through TLR4 activation, the mechanism of Ang II-induced renal inflammatory reaction in MCs mainly relies on the crosstalk between Ang II and TLR4 signal. Similar mechanisms are supported by other studies on the liver fibrosis model or vascular smooth muscle cells [33,34].

We provided evidence that Ang II contributes to increased mesangial inflammatory stress via the innate immune system, and documented the possibility that Ang II has synergistic effects on the release of proinflammatory factors via TLR4 signaling in rat MCs with hyperglycemia, TLR4 signal plays a pivotal role in this pathogenesis and cross-talk between Ang II and TLR4 contributed to the MC inflammation under HG conditions. Irbesartan prevented the release of inflammatory cytokines partly through attenuating TLR4 signaling in MCs under HG conditions. This may be a promising intervention strategy for blocking the inflammatory reaction and effectively preventing diabetic kidney disease through controlling TLR4 signaling combined with an RAAS blockade [35,36].

Acknowledgments

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Conflict of interest

The authors declare that there are no conflicts of interest.

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