Angiotensin II Increases Oxidative Stress and Induces Glomerular Sclerosis via Toll-like Receptor 4

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Abstract  Background: Angiotensin II (AngII) increases reactive oxygen species (ROS) and induces glomerular sclerosis. Toll-like receptor 4 (TLR4)-mediated inflammation enhances the renal impairment in renal inflammatory diseases. The relationship between TLR4 and AngII-induced glomerular sclerosis is unknown. Methods: Mice lacking TLR4 function (Tlr4<sup>lps-d</sup>) and wild-type (WT) mice were randomized into groups treated with AngII, norepinephrine (NE) or a sub-depressor dose of the AngII receptor blocker irbesartan along with AngII for 2 weeks. We then assessed the expressions of NADPH oxidase and monocyte chemoattractant protein-1 (MCP-1) and the inflammatory cell recruitment in the glomeruli. We also evaluated the mesangial matrix proliferation and ROS. Results: AngII and NE equally increased the systolic blood pressure compared to the control mice (<i>p</i>&lt;0.05). In the WT mice treated with AngII, we observed glomerular sclerosis, an increase in NADPH oxidase, MCP-1 and the infiltration of macrophages as well as ROS content in the glomeruli compared to the control mice (<i>p</i>&lt;0.05), whereas the Tlr4<sup>lps-d</sup> mice showed little effects of AngII on these indices. In addition, the sub-depressor-dose irbesartan treatment reversed these changes. NE had little effects on these indices. Conclusions: TLR4 plays an important role in AngII-induced oxidative stress, inflammation and glomerular sclerosis through the AT1 receptor.

Key words: glomerular sclerosis, oxidative stress, inflammation, monocyte chemoattractant protein-1, angiotensin II

Introduction

Activation of the renin-angiotensin (Ang) system plays an important role in the pathological mechanisms of target organ damage and the progression of hypertension. Specifically, direct renal effects of angiotensin II (AngII) contribute to the pathogenesis and progression of glomerular sclerosis in hypertension through activated oxidative stress
and inflammation.\textsuperscript{3,4} The Toll-like receptor (TLR) family is a major component of pathogen-associated molecular pattern-recognition molecules,\textsuperscript{5} and members of this family mediate signal transduction pathways through the activation of transcription factors that regulate the expression of proinflammatory cytokines and chemokines. TLRs have a central role in innate immunity.\textsuperscript{6} In addition, the majority of the constitutive TLR4 expression in the kidney is found in tubular epithelial cells, glomerular endothelial cells, and podocytes,\textsuperscript{7} and TLR4 might have an important role in the pathogenesis of a variety of renal diseases.\textsuperscript{8}

Both AngII and TLR4 activate NADPH oxidase to produce reactive oxygen species (ROS), which is followed by the activation of proinflammatory transcription factors such as nuclear factor-κB (NF-κB); they stimulate the expression of monocyte chemoattractant protein-1 (MCP-1) and may be involved in the development of hypertension.\textsuperscript{9-11} However, it remains unknown whether TLR4 presents in the kidney modulates AngII signal transduction, and whether TLR4 is involved in the proinflammatory process of AngII. In the course of efforts to identify strategies to prevent TLR-mediated local inflammation, the previous studies results concerning the role of TLRs in renal diseases are conflicting.\textsuperscript{8} In the present study, to clarify whether TLR4 is involved in the glomerular sclerosis induced by AngII stimulation, we investigated the effects of TLR4 on oxidative stress, inflammation, and glomerular sclerosis in AngII-induced hypertension.

\textbf{Materials and methods}

\textbf{Animals and experimental protocols}

We randomized male 12- to 16-week-old wild-type (WT) mice (BALB/c, n=24; Charles River Japan, Japan) and congenic homozygous TLR4-deficient mice on a BALB/c background (C3H-Tlr4\textsuperscript{−/−}, The Jackson Laboratory, USA; Tlr4\textsuperscript{−/−} mice; n=24)\textsuperscript{12,13} into four groups, treated with physiological saline (the control group; n=6), AngII (Sigma-Aldrich, USA; 1.1 mg·kg\textsuperscript{−1}·day\textsuperscript{−1}, the AngII-treated group; n=6), norepinephrine (5.6 mg·kg\textsuperscript{−1}·day\textsuperscript{−1}, the NE-treated group; n=6), or the sub-depressor dose Irbesartan (IRB), a selective AT1 receptor antagonist, (Shionogi Pharmaceutical, Tokyo, Japan) with AngII (IRB 6 mg·kg\textsuperscript{−1}·day\textsuperscript{−1}, the IRB-treated group; n=6). The doses used in the experiments were previously reported.\textsuperscript{14}

An osmotic minipump (Model 1002; ALZET Osmotic Pumps, USA) was implanted under the anesthesia with isoflurane (Abbott Japan, Tokyo, Japan). The physiological saline, AngII, and NE were administered subcutaneously by the osmotic minipump for 2 weeks. IRB was given in drinking water along with the AngII treatment by minipump for 2 weeks. We evaluated the kidney weights and body weights of the mice after the 2-week treatment, and the systolic blood pressure and heart rate were measured by tail-cuff plethysmography without anesthesia on the 7th and 14th days during the experiment.

The Ethics Committee for Animal Experimentation at the Yamaguchi University School of Medicine approved the experimental protocol used in this study. The study also conformed to the Yamaguchi University’s Guidelines for the Care and Use of Laboratory Animals.

\textbf{Tissue preparation}

After the 2-week treatment period, the mice were euthanized under inhalation anesthesia with an excessive dose of isoflurane. The kidneys were immediately separated, washed with phosphate-buffered saline, and weighed. A piece of the middle portion of the kidney tissue from each mouse was snap-frozen with optimal cutting temperature compound in liquid nitrogen to obtain fresh-frozen, 5-μm-thick sections for dihydroethidium (DHE) staining. The other middle portion of the kidney, which was fixed in 95% ethanol and 1% glacial acetic acid for immunohistochemistry,\textsuperscript{15} was paraffin-embedded and sectioned into 2-μm slices for the evaluation of the expressions of p22\textsuperscript{phox}, p47\textsuperscript{phox} and MCP-1 and for a morphology assessment using hematoxylin staining.

\textbf{Histological and immunohistochemical analyses}

In each mouse, the percent global glomerular sclerosis was measured as described.\textsuperscript{16}
Briefly, sections were stained with periodic acid-Schiff (PAS) reagent and counterstained with Hematoxylin, and then 50 glomeruli were used for the measurement of the percent global glomerular sclerosis excluding the glomeruli present in the cortical outermost portions of each kidney tissue sample, to avoid the influence of pressure on the sample. The sections were quantified morphometrically with a camera control program system (ACT-1, ver. 2.51) with a digital camera (DX-M1200F; Nikon, Tokyo) connected to an automated microscope (Eclipse E1000, Nikon).

The immunohistochemical analyses used rabbit polyclonal antibody against human MCP-1 (Cell Signaling Technology, USA); goat polyclonal antibodies against the human membrane-bound flavocytochrome-containing subunit, p22^{phox}, and the cytosolic component, p47^{phox}, of the NADPH oxidase (Santa Cruz Biotechnology, USA) and rat monoclonal antibody against mouse macrophage marker BM8 (BMA Biomedicals, Switzerland) for the expressions of p22^{phox}, p47^{phox}, MCP-1 and macrophages marker BM8 in the glomerulus of the kidney tissue by the avidin-biotinylated enzyme complex method with serial sections (Vector Laboratories, USA). The total number of BM8-positive macrophages in five randomly selected glomeruli in each mouse was counted in a blinded manner in randomly selected high-power fields per tissue section from four mice per group. The mean values for each kidney were used for the statistical analysis.

In situ evaluation of ·O\textsubscript{2}− in the glomeruli
Unfixed, frozen, 5-μm-thick kidney segments were prepared for the in situ imaging of ROS generation in the glomeruli. We evaluated the ·O\textsubscript{2}− content using fluorescent DHE (2 μmol/L; Polysciences, USA) in a blinded manner as described. The mean values for each kidney were used for the statistical analysis. These data are expressed as a fold increase of the corresponding data for the control WT mice.

Statistical analysis
All of the values are expressed as the mean ± standard error (SE). We compared the experimental groups using an analysis of variance (ANOVA) followed by Scheffe’s multiple comparisons using StatView software, ver. 5.01, (SAS, USA). P-values <0.05 were accepted as significant.

Results

Body weight, blood pressure and kidney weight
Throughout the present experiments, the control WT and Tlr4\textsuperscript{lps-d} mice showed similar body weights and heart rates, and the drug treatments did not affect these indices. As shown in Table 1, AngII and NE similarly and significantly increased the systolic blood pressure in all drug-treated groups compared to the control WT and Tlr4\textsuperscript{lps-d} mice. Little difference in blood pressure was observed among the drug-treated groups. The administration of AngII, IRB or NE did not affect the kidney weight/body weight ratios compared to the control WT and Tlr4\textsuperscript{lps-d} mice (Table 1).

TLR4 deficiency inhibited the AngII-induced glomerular sclerosis
Representative figures of glomerular sclerosis assessed by PAS stain are shown in Figure 1A. AngII increased the percent glomerular sclerosis in the cortical portion of the kidney in the WT mice, whereas the Tlr4\textsuperscript{lps-d} mice showed little effect of AngII with respect to this index compared to the WT mice. In both the WT and Tlr4\textsuperscript{lps-d} mice, although the percent glomerular sclerosis in the AngII and NE-treated groups was slightly higher than those of the control WT and Tlr4\textsuperscript{lps-d} mice, the differences were not significant among the control WT and Tlr4\textsuperscript{lps-d} mice, and the treatment with the AT1 receptor antagonist IRB inhibited the AngII-induced glomerular sclerosis in the AngII-treated WT mice (Fig. 1B).

TLR4 deficiency abolished the AngII-induced ROS in the glomeruli
Figure 2A provides representative figures of the DHE staining used to assess the ·O\textsubscript{2}− content in the glomerulus in the kidney. The control WT and Tlr4\textsuperscript{lps-d} mice showed minimal fluorescence in the glomerulus in the kidney. In the quantitative analysis, the AngII-treated WT mice showed twofold higher values for DHE fluorescence compared to the control WT mice, reflecting increased
\({\text{O}_2^-}\) content in the glomerulus of the mouse kidney, and IRB significantly reduced the DHE density compared to that in the AngII-treatment alone group (Fig. 2B).

In contrast, the AngII-induced increase in the DHE signal was markedly suppressed in Tlr4\(^{lps-d}\) mice compared to that in the AngII-treated WT mice, whereas it was not affected by the IRB treatment. NE slightly increased the DHE signal in the kidney in both the WT and Tlr4\(^{lps-d}\) mice, and the signal was lower than that in the AngII-treated WT mice, but the differences did not reach significance among the other groups (Fig. 2B).

**TLR4 deficiency inhibited the AngII-induced increase in the expression of NADPH oxidase components in the glomeruli**

To clarify whether TLR4 affects the NADPH oxidase expression in the glomeruli of the cortical portion of the kidney, we examined the effects of TLR4 on the expressions of both the membrane-bound subunit p22\(^{phox}\) and the cytosolic component p47\(^{phox}\) of the NADPH oxidase in the WT and Tlr4\(^{lps-d}\) mice. We performed the immunohistochemistry for the expressions of p22\(^{phox}\) and p47\(^{phox}\) in the cortical portion of the kidney tissue (Fig. 3A). Immunohistochemically, both p22\(^{phox}\) and p47\(^{phox}\) were positively stained with antibodies against p22\(^{phox}\) and p47\(^{phox}\), and were specifically and heterogeneously stained mainly in the mesangial cells and podocytes in the glomerulus in the kidney of both the WT and Tlr4\(^{lps-d}\) mice (brown against a pale blue background, respectively; Fig. 3A). The quantitative analysis showed that AngII significantly increased the expression of both p22\(^{phox}\) and p47\(^{phox}\) by 3-4-fold compared to the levels in the control WT mice. Either TLR4 deficiency or the treatment with a sub-depressor dose of IRB abolished the AngII-induced increases in the expression of both p22\(^{phox}\) and p47\(^{phox}\) (Fig. 3B). In the NE-treated group, the WT and Tlr4\(^{lps-d}\) mice showed little increase in the expressions of p22\(^{phox}\) and p47\(^{phox}\) in the glomeruli in the kidney, with little difference from that in the control WT and Tlr4\(^{lps-d}\) mice (Fig. 3B).

**TLR4 deficiency abolished the AngII-induced infiltration of macrophages and the up-regulation of MCP-1 expression in the glomeruli**

Immunohistochemically, macrophages were stained red against a pale blue background, mainly in the mesangial area of the mouse glomeruli (Fig. 4A). The quantitative analysis

<table>
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<th>Table 1</th>
<th>Body weight, kidney weight/body weight, systolic blood pressure and heart rate on the 14th day of the experiment.</th>
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<tr>
<td></td>
<td>WT</td>
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<td>Body weight, g</td>
<td>28.5±0.6</td>
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<td>Left kidney weight/body weight</td>
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<td>mg/g</td>
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<tr>
<td>Right kidney weight/body weight</td>
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<tr>
<td>mg/g</td>
<td>500±46</td>
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<tr>
<td>Systolic blood pressure, mm Hg</td>
<td></td>
</tr>
<tr>
<td>Heart rate, beats/minutes</td>
<td>528±38</td>
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WT; wild-type mice, Tlr4\(^{lps-d}\); TLR4 deficient mice, AngII; angiotensin II, NE; norepinephrine, IRB; irbesartan. Values are means ± SE. Number of each group=5. *P<0.05 vs. control WT mice, † P<0.05 vs. control Tlr4\(^{lps-d}\) mice.
Fig. 1 Representative micrograph of the glomerular sclerosis of the kidney. WT: wild-type mice, Tlr4<sup>lps-d</sup>: TLR4-deficient mice, AngII: angiotensin II, NE: norepinephrine, IRB: irbesartan. A: Representative micrographs of the effects of TLR4 on glomerular sclerosis in the mouse kidney tissue. Sections were stained with PAS staining. Bar, 50 μm. B: Quantitative analysis of glomerular sclerosis in the kidney. Bar, SE. Experiments, n=5. *p<0.05 vs. the control WT and Tlr4<sup>lps-d</sup> groups.

Fig. 2 ROS content in the glomerulus of the kidney. A: Representative fluorescent photomicrographs showing the in situ detection of ·O<sub>2</sub>· in the glomerulus of the mouse kidney tissue labeled with oxidative dihydroethidium (DHE; red fluorescence). Bar, 100 μm. B: Results of a quantitative analysis of ·O<sub>2</sub>· content. Bar, SE. Experiments, n=3. *p<0.05 vs. the control WT and Tlr4<sup>lps-d</sup> groups.
showed that, in the WT mice, the infiltration of macrophages in the glomeruli was remarkably increased by 7.5-fold following AngII treatment compared to the control WT mice, and this was suppressed with the sub-depressor-dose IRB treatment. NE treatment did not affect the increase in the infiltration of macrophages in the glomeruli compared to the controls in both the WT and Tlr4 lps-d mice (Fig. 4B).

Immunohistochemically, MCP-1 was stained specifically and heterogeneously, mainly in the cytoplasm of the mesangial cells and podocytes in the glomeruli in the kidney of both the WT and Tlr4 lps-d mice (brown against a pale blue background, respectively; Fig. 5A). The quantitative analysis showed similar levels of expression in the glomeruli of the kidney in the control WT and Tlr4 lps-d mice for MCP-1 (Fig. 5B). In the WT mice, the expression of MCP-1 in the glomeruli was increased by 2.5-fold with AngII treatment compared to the controls, and this expression was suppressed with IRB treatment, whereas the MCP-1 expression in the glomeruli of the Tlr4 lps-d mice was unaffected by AngII or IRB (Fig. 5B). NE treatment resulted in little increase in the expression of MCP-1 in the glomeruli of the kidney compared to the controls among both the WT and Tlr4 lps-d mice (Fig. 5B).

**Discussion**

Our results are in agreement with the previous report that AngII is important for the generation of ROS, the infiltration of macrophages in the glomeruli and glomerular sclerosis independent of blood pressure. Important findings of the present study is that TLR4 plays an important role in the progression of the glomerular sclerosis in the kidney induced by AngII treatment.

In the kidney, TLR4 plays a pivotal role within the innate immune system which contributes to the host defense against exogenous microbial pathogens by detecting exogenous ligands. TLR4 ligands can promote renal injury by increasing inflammation, independently of any effects of the adaptive immune response. It was also suggested that TLR signaling predominates in intrinsic renal cells rather than leukocytes, and TLR4 has been shown to be stimulated by endogenous ligands during inflammation and oxidative stress. Our present findings agree
with these reports, suggesting that TLR4 is involved in the process by which AngII induces ROS and increases inflammation in the glomerulus.

It has been reported that TLR4-deficient mice showed fewer granulocytes and a smaller amount of chemokine infiltrate in the kidneys compared to wild-type mice. We also reported that AngII induced cardiac hypertrophy and dysfunction as well as vascular remodeling and perivascular fibrosis and monocyte/macrophage infiltration through TLR4 by increasing oxidative stress and selective activation of MCP-1 in the heart in cases of AngII-induced hypertension. In addition, it was reported that AngII stimulated

Fig. 4  Infiltration of macrophages in the glomerulus of the kidney.
A: Immunohistochemical staining of macrophages in the glomeruli in mouse kidney tissue. Tissue macrophages were stained brown in the glomerulus of the kidney. Bar, 50 μm. B: Results of a quantitative analysis of the macrophage count in the glomerulus of the kidney. Bar, SE. Experiments, n=4. *p<0.05 vs. the other groups.

Fig. 5  Expression of MCP-1 in the glomerulus of the kidney.
A: Immunohistochemical staining of MCP-1 in the mouse kidney tissue. MCP-1 was stained brown in the cytoplasm in the glomerulus of the kidney. Bar, 50 μm. B: Results of a quantitative analysis of the expression of MCP-1 in the glomerulus of the kidney. Bar, SE. Experiments, n=3. *p<0.05 vs. the other groups.
the transcription of TLR4 mRNA in mesangial cells, resulting in an up-regulation of TLR4 protein with enhanced NF-κB signaling and the induction of chemokines, suggesting that AngII contributes to inflammation by modifying innate immunity. In the present study, we also observed that TLR4 modified the ROS production and inflammation in the glomeruli by the induction of macrophages infiltrating into the glomeruli by AngII stimulation.

NADPH oxidase, a major source of ROS, is a critical mediator of redox signaling, and oxidative stress is associated with the development of glomerular diseases. The renin-angiotensin system was reported to be crucial for NADPH oxidase-dependent redox signaling pathways, which provoke the upregulation of MCP-1, and it was shown that this process is blunted by an AT1 receptor antagonist. This suggests that TLR4’s function may be essential for the up-regulation of NADPH oxidase-dependent ROS signaling and the inflammatory process, followed by glomerular sclerosis induced by AngII administration as shown in the present study. The results of the present study agree with these previous reports, showing that a TLR4 signaling pathway may play an essential role in the NADPH oxidase-dependent ROS-generating redox signaling pathway following macrophage infiltration, inflammation and glomerular sclerosis in the kidney.

It has been shown that AngII-induced hypertension is ROS dependent. In contrast, we found that Ang II-induced blood pressure in TLR4 deficient mice is similar to WT mice, although Ang II induced increase in ROS level is reduced. Reasons for these discrepancies are unclear, but may relate to differences of genetic background (C57Bl/6 versus Balb/c) and duration of exposure to either increased ROS or AngII: i.e., lifetime (genetic model) versus several weeks (infusion model). Indeed, there are conflicting results about the role of AngII-generated ROS actions in hypertension.

We did not examine the effects of AngII on the expressions of AT1 receptor and Ang-converting enzyme in the kidney in the present study, but based on our present findings, we suggest that the AT1 receptor signaling pathway may be involved in glomerular sclerosis, and that the blood pressure level may be dependent on the AT1 receptor signaling pathway; TLR4 may be necessary to produce oxidative stress in the kidney by Ang stimulation. Further research is necessary to confirm the expressions of Ang receptor and Ang-converting enzyme in the kidney.

Second, apart from AngII, aldosterone exerts a pro-oxidant action mediated through different pathways, including NADPH oxidase-dependent mechanisms. The pro-inflammatory and pro-fibrotic effects of aldosterone were proposed to be mediated by NF-κB activation, a factor involved in inflammation, immunity, cell proliferation, and aldosterone that may cause renal fibrosis and glomerulosclerosis. This process needs to be clarified in future research.

In conclusion, our present findings demonstrated that although hypertension induces mild glomerular sclerosis irrespective of the existence of TLR4, AngII increases oxidative stress through the AT1 receptor under the existence of TLR4, and further promotes the inflammation and glomerular sclerosis. These data provide the first evidence of a causal role of TLR4 in AngII-induced glomerular sclerosis. TLR4 inhibition may therefore constitute a novel therapeutic approach to inhibiting hypertensive glomerular sclerosis following chronic kidney disease in patients with hypertension.

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Conflicts of Interest

S.U. received a consultant fee from Boehringer Ingelheim Japan, Inc., honoraria from Kyowa Hakko Kirin Co., Ltd., Takeda Pharmaceutical Co., Ltd., Mochida Pharmaceutical

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