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ORIGINAL ARTICLE

TLR4 is a critical regulator of angiotensin II-induced vascular remodeling: the roles of extracellular SOD and NADPH oxidase

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Toll-like receptor 4 (TLR4) and angiotensin II (AngII) induce vascular remodeling through the production of reactive oxygen species (ROS). AngII has also been shown to increase antioxidant enzyme extracellular superoxide dismutase (ecSOD). However, the roles of TLR4 in Ang II-induced ROS production, vascular remodeling and hypertension remain unknown. Mice lacking TLR4 function showed significant inhibition of vascular remodeling in response to chronic AngII infusion, with no impact on blood pressure. The increases in ROS level and NADPH oxidase activity in response to AngII infusion were markedly blunted in TLR4-deficient mice. Similar effects were observed in wild-type (WT) mice treated with a sub-depressor dose of the AT1 receptor antagonist irbesartan, which had no effects on TLR4-deficient mice. Intriguingly, the AngII infusion-induced increases in ecSOD activity and expression were rather enhanced in TLR4-deficient mice compared with WT mice, whereas the expression of the proinflammatory chemokine MCP-1 was decreased. Importantly, AngII-induced vascular remodeling was positively correlated with NADPH oxidase activity, ROS levels and MCP-1 expression levels. Notably, chronic norepinephrine infusion, which elevates blood pressure without increasing ROS production, did not induce significant vascular remodeling in WT mice. Taken together, these findings suggest that ROS elevation is required for accelerating vascular remodeling but not for hypertensive effects in this model. We demonstrated that TLR4 plays a pivotal role in regulating AngII-induced vascular ROS levels by inhibiting the expression and activity of the antioxidant enzyme ecSOD, as well as by activating NADPH oxidase, which enhances inflammation to facilitate the progression of vascular remodeling.
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Keywords: angiotensin II; extracellular superoxide dismutase; Toll-like receptor 4; vascular remodeling

INTRODUCTION

In hypertension, angiotensin II (AngII) generates reactive oxygen species (ROS) by the activation of NADPH oxidase through AngII type 1 (AT1) receptors, which mediate many of the actions of AngII, including constriction of vascular smooth muscles, increased systemic blood pressure, endothelial dysfunction, inflammation and vascular remodeling.[1–3] Concomitantly, AngII also upregulates the expression and activity of a major antioxidant enzyme in the vascular extracellular space: extracellular superoxide dismutase (ecSOD).[4,5] Indeed, mice lacking NADPH oxidase showed blunted AngII-induced hypertension,[6,7] whereas mice lacking ecSOD showed enhanced AngII effects.[8,9] However, there have been conflicting results regarding the role of AngII-generated ROS actions on various vascular consequences, including hypertension, hypertrophy and inflammation.[1,2]

Toll-like receptors (TLRs) have been primarily identified as the primary innate immune receptors.[10,11] However, it has recently been reported that TLR recognition is not restricted to exogenous microbial patterns, and putative endogenous TLR ligands might be released during tissue damage, driving inflammation in the absence of infection.[12,13] In addition, there has been growing evidence showing the contributions of TLR signaling pathways to inflammation and the progression of cardiovascular disease.[11,14,15] Several studies have shown that some TLR types are expressed on the arterial wall,[11,16,17] and in particular, TLR4 accelerates vascular remodeling and atherosclerosis at the vessel wall.[15,18] It has also been reported that TLR4 is involved in lipopolysaccharide (LPS)-induced ROS via interaction with NADPH oxidase.[19,20] These findings suggest that TLR4 might have an association with AngII-derived ROS regulation and vascular remodeling.

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However, it remains unknown how TLR4 is involved in ROS regulation at the vessel wall and how it modulates vascular remodeling in AngII-induced hypertension. In this study, we examined the relationships among TLR4, AT1 receptors, NADPH oxidase-derived ROS, antioxidant enzyme ecSOD and inflammation in vascular remodeling in AngII-induced hypertension.

METHODS

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

Chemicals and antibodies

AngII acetate hydrate, L-(-)-norepinephrine bitartrate salt monohydrate and SOD-mimetic 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (Tempol) were purchased from Sigma-Aldrich (St Louis, MO, USA). Irbesartan (IRB), a selective AT1 receptor antagonist, was kindly provided by Shionogi Pharmaceutical (Tokyo, Japan). The following antibodies were applied for immunohistochemistry or immunoblots: rabbit polyclonal antibodies against mouse monocyte chemoattractant protein-1 (MCP-1) (Cell Signaling Technology, Boston, MA, USA); rabbit polyclonal antibodies against human Cu/ZnSOD (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and goat polyclonal antibody against human α-smooth muscle actin (SMA, DAKO Japan, Kyoto, Japan). Affinity-purified mouse ecSOD antibody was prepared as previously reported.[5,21]

Experimental protocol

Male 12–16-week-old male wild-type (WT) mice (Balb/c, Charles River Japan, Yokohama, Japan, WT mice; $n=12$) and homozygous TLR4-deficient mice on a Balb/c background ($Tlr4^{lps\text{-}d}$, The Jackson Laboratory, Bar Harbor, ME, USA; $Tlr4^{lps\text{-}d}$ mice; $n=12$)[22–24] were maintained at the Science Research Center of Yamaguchi University.

The WT and $Tlr4^{lps\text{-}d}$ mice were randomized into four groups each and were treated with physiologic saline (control group), AngII (1.1 mg kg^{-1} per day, AngII-treated group), norepinephrine (5.6 mg kg^{-1} day, NE-treated group) and a sub-depressor dose of IRB with AngII (IRB 6 mg kg^{-1} per day, IRB-treated group). In some of the experiments, a high dose of IRB (21.2 mg kg^{-1} per day) was used. The doses used in the experiments were determined by preliminary experiments. Anesthesia of the mice was induced with isoflurane (Abbott Japan, Tokyo, Japan) at a concentration of 3% and then was maintained at 1% by a face mask during the implantation of osmotic minipumps (Model 1002; Alzet Osmotic Pumps, Cupertino, CA, USA). Physiologic saline, AngII and NE were administered subcutaneously by osmotic minipumps for 2 weeks. IRB was provided in drinking water with AngII treatment for 2 weeks. We evaluated body weight after the 2-week treatment, and systolic blood pressure and heart rate were measured by tail-cuff plethysmography without anesthesia on the seventh and fourteenth days of the experiment.

Tissue preparation, histological and immunohistochemical analyses

After the 2-week treatment, the mice were killed under inhalation anesthesia with an excessive dose of isoflurane. In each mouse, the proximal portion of the aorta was snap-frozen with an optimal cutting temperature compound in liquid nitrogen to obtain fresh-frozen, 30-μm-thick sections for dihydroethidium (DHE) staining.[25] The remainder of the specimens, fixed in 10% buffered formaldehyde, were paraffin-embedded and were sectioned into 4-μm slices for the evaluation of vascular morphology using Masson's trichrome or hematoxylin and eosin staining. The remaining aortic tissues were kept at −80 °C for other experiments.

The sections were quantified morphometrically with a camera control program system (ACT-1, version 2.51, Nikon, Tokyo, Japan), using a digital camera (DXM1200F, Nikon) connected to an automated microscope (Eclipse E1000, Nikon). We assessed the wall-to-lumen ratio and the total cell number in the tunica media ($\times 10^3$ mm^{-2}) of the aorta, as previously reported.[26–28] Briefly, the aorta was examined at three points of the cross-sectional area in a blind manner, using at least two slices for each aorta with NIH Image software, version 1.62 (Bethesda, MD, USA). The mean value of each aorta was used for statistical analysis. Selective and quantitative analyses for aortic morphology and protein expression were performed as previously described.[26,27] Immunohistochemistry for the expression of MCP-1 and TLR4 in the vascular wall was performed using the avidin-biotinylated enzyme complex method with serial sections (Vector Laboratories, San Francisco, CA, USA).[26]

Detection of NADPH oxidase activity and ecSOD activity

The activity of NADPH oxidase activity in the aorta was measured by luminoassay with 5 μM lucigenin (bis-*N*-methylacridinium nitrate) as the electron acceptor and 100 μM NADPH as the substrate, as previously reported.[25] The lucigenin count was expressed as counts per minute per microgram of the tissue homogenate.

ConA-Sepharose chromatography (Pharmacia Biotech, Piscataway, NJ, USA) was used to isolate ecSOD as previously described.[25,29,30] The activity of ecSOD was measured using an SOD assay kit (R&D Systems, Minneapolis, MN, USA).[25]

Detection of $\cdot O_2^-$ in the vascular wall

Unfixed, frozen, 30-μm-thick aortic segments were prepared for *in situ* imaging of ROS generation in the vascular wall. We evaluated $\cdot O_2^-$ content with fluorescent DHE (Polysciences, Warrington, PA, USA) as previously described.[26] The specificity of DHE signals for $\cdot O_2^-$ detection was confirmed by preincubation with SOD-mimetic Tempol (500 U ml^{-1}). The images were obtained with a laser scanning confocal microscope (LSM510, Zeiss, Munich, Germany). The cellular sites of $\cdot O_2^-$ production in both the intima and media, which were determined with HE-stained serial sections, were assessed in a blind manner. These data are expressed as percentages of the corresponding data for the control WT mice.

Immunoblotting

Proteins were extracted from the frozen mouse aortic tissues homogenized in 25 mM Tris (pH 7.4), containing 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, 10 mM sodium pyrophosphate, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM phenylmethane sulfonyl fluoride and 10 mg ml^{-1} aprotinin, with Triton X-100 added to a final concentration of 1% to extract the protein. The protein concentrations were determined using a BCA protein assay kit (Bio-Rad, Hercules, CA, USA). For determining each protein expression, equal amounts of sample proteins were loaded in each lane of a sodium dodecyl sulfate polyacrylamide gel, transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) and probed with rabbit polyclonal antibodies against human Cu/ZnSOD (Santa Cruz Biotechnology), affinity-purified mouse ecSOD antibody and goat polyclonal antibody against human α-SMA (DAKO Japan, Kyoto, Japan). The target proteins were visualized and determined with an enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, UK) using NIH Image software, version 1.62, as described previously.[28]

Statistical analysis

All of the values are expressed as the mean ± s.e. The experimental groups were compared with analysis of variance followed by Scheffe's multiple comparisons, using StatView software, version 5.01 (SAS Institute, Cary, NC, USA). The data for the relationships among wall-to-lumen ratio, fold increase in $\cdot O_2^-$ content and MCP-1, and NADPH oxidase and ecSOD activity were statistically analyzed by simple linear regression analysis. Values of $P<0.05$ were considered statistically significant.

RESULTS

TLR deficiency prevents AngII-induced vascular remodeling

Table 1 summarizes the data for body weight, heart rate, systolic blood pressure and the total cell numbers in the tunica media of mice on the fourteenth day in the experiments. The control WT and $Tlr4^{lps\text{-}d}$ mice showed similar body weights and heart rates, and drug treatment did not affect these indices. AngII and NE similarly and significantly increased systolic blood pressure in all of the drug-treated groups

Table 1 Body weight, heart rate, systolic blood pressure and vascular remodeling on the fourteenth day of the experiment

	WT				*Tlr4$^{lps\text{-}d}$*			
	Control	*AngII*	*IRB*	*NE*	*Control*	*AngII*	*IRB*	*NE*
Body weight, g	28.9±0.5	27.3±1.1	27.0±1.3	27.6±1.5	29.0±1.0	30.0±2.9	26.8±1.4	26.9±1.9
Heart rate, beats per minutes	476±64	479±42	510±25	492±33	513±17	415±54	473±40	482±20
Systolic blood pressure, mm Hg	105±3	169±3[a]	157±2[a]	158±9[a]	115±4	173±3[b]	158±8[b]	160±7[b]
Total cell number, $\times 10^3$ mm^{-2}	4813±309	5103±94	4828±131	4953±407	4680±120	4752±209	4895±480	4937±388

Abbreviations: AngII, angiotensin II; NE, norepinephrine; IRB, irbesartan; Tlr4$^{lps\text{-}d}$, TLR4 deficient mice; WT, wild-type mice. A quantitative analysis of total cell number in the tunica media of the abdominal aorta was evaluated in hematoxylin and eosin staining. Values are means±s.e. Number of each group=3.
[a]$P<0.05$ *vs.* control WT mice.
[b]$P<0.05$ *vs.* control Tlr4$^{lps\text{-}d}$ mice.

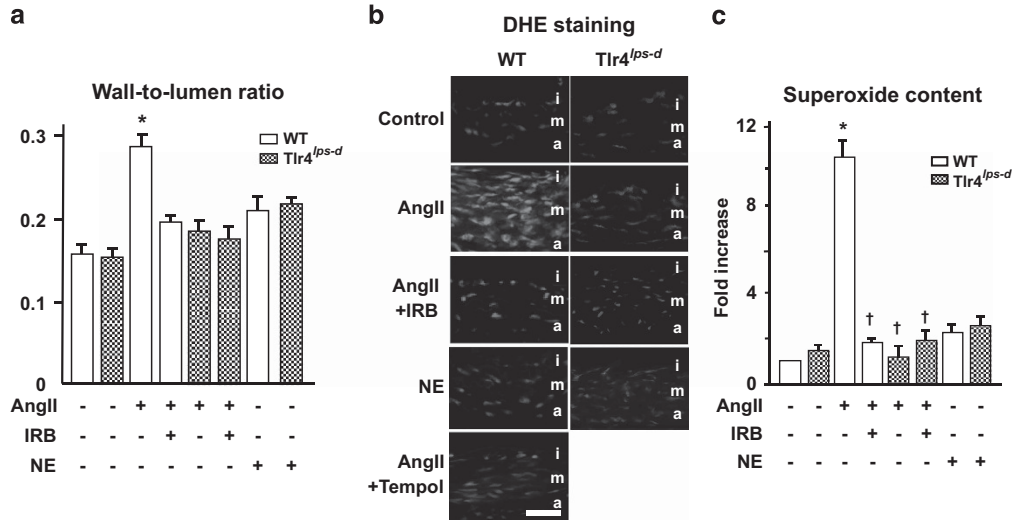

Figure 1 Vascular remodeling and *in situ* detection of superoxide ($\cdot O_2^-$) of the abdominal aorta. (**a**) Results of a quantitative analysis of the wall-to-lumen ratio. Bars indicate s.e. Experiments, $n=3$. *$P<0.05$ *vs.* the other groups. (**b**) Fluorescent photomicrographs showing *in situ* detection of $\cdot O_2^-$ in the aorta labeled with oxidative DHE (red fluorescence). Bar, 50 μm. Note the increased fluorescence reflecting $\cdot O_2^-$ levels in the intima (i), media (m) and adventitia (a) of the AngII-treated WT mice. The specificity of DHE signals for $\cdot O_2^-$ detection was confirmed by preincubation with SOD-mimetic Tempol. Photographs are representative of three independent experiments. DHE, dihydroethidine. (**c**) Results of a quantitative analysis of the proportion of DHE fluorescence. Bars indicate s.e. Experiments, $n=3$. *$P<0.05$ *vs.* the other groups. AngII, angiotensin II; IRB, irbesartan; NE, norepinephrine; Tlr4$^{lps\text{-}d}$, TLR4-deficient mice; WT, wild-type. A full color version of this figure is available at *Hypertension Research* online.

compared with the control WT and Tlr4$^{lps\text{-}d}$ mice. Little difference in blood pressures was observed among the drug-treated groups. The control WT and Tlr4$^{lps\text{-}d}$ mice showed similar levels of total cell numbers in the aortic tunica media. Furthermore, the total cell numbers in the tunica media were unchanged by any drug treatment in both the WT and Tlr4$^{lps\text{-}d}$ mice.

The control WT and Tlr4$^{lps\text{-}d}$ mice showed similar levels of wall-to-lumen ratio, as shown in Figure 1a. In WT mice, AngII induced a significant increase in the wall-to-lumen ratio compared with that in the control mice. However, in the Tlr4$^{lps\text{-}d}$ mice, AngII did not increase the wall-to-lumen ratio compared with the values in the control mice. In the WT mice, treatment with the AT1 receptor blocker IRB significantly rescued the increase in the wall-to-lumen ratio compared with AngII treatment alone. In the NE-treated groups, both WT and Tlr4$^{lps\text{-}d}$ mice showed little increase in the wall-to-lumen ratio compared with that in the control mice.

TLR4 deficiency abolishes AngII-induced vascular ROS

Figure 1b shows *in situ* detection of $\cdot O_2^-$ in the abdominal aorta. The WT and Tlr4$^{lps\text{-}d}$ mice showed minimal DHE fluorescence in the intima and adventitia, and only a slight signal of DHE fluorescence was noticed in the aortic media. As shown in Figure 1c, quantitative analysis demonstrated that AngII-treated WT mice showed 10-fold higher values in $\cdot O_2^-$ content assessed by DHE density throughout the vessel wall, with the highest DHE signal in the media compared with that in the control WT mice, whereas a sub-depressor dose of IRB significantly reduced the DHE density compared with the AngII treatment alone. In contrast, few effects of IRB on the DHE signals in the vascular wall of the aorta were observed in the AngII-treated Tlr4$^{lps\text{-}d}$ mice compared with the AngII-treated WT mice. By co-incubation with Tempol, the increase in DHE density in the vascular wall in the AngII-treated WT mice was markedly decreased, suggesting that DHE signaling mainly reflected an increase in $\cdot O_2^-$ (Figure 1b). In the both WT and Tlr4$^{lps\text{-}d}$ mice, NE treatment resulted in only a slight increase in the $\cdot O_2^-$ content in the vascular wall.

TLR4 deficiency inhibits AngII-induced vascular NADPH oxidase activity in the aorta

AngII significantly increased (by threefold) vascular NADPH oxidase activity in only the WT mice (Figure 2). Treatment with a sub-depressor dose of IRB prevented the increase in NADPH oxidase activity in the aorta of WT mice by AngII treatment. In the NE-treated

groups, the WT and Tlr4$^{lps\text{-}d}$ mice showed a slight increase in NADPH oxidase activity in the vascular wall, with little difference from that in the control WT and Tlr4$^{lps\text{-}d}$ mice.

TLR4 deficiency abolishes AngII-induced MCP-1 expression in the aorta

Immunochemically, MCP-1 was stained brown in the cytoplasms of the endothelium and medial cells (Figure 3a). Quantitative analysis showed similar levels of expression in the vascular wall in the control WT and Tlr4$^{lps\text{-}d}$ mice for MCP-1 (Figure 3b). In the WT mice, the expression of MCP-1 in the vascular wall was increased by 3.5-fold with AngII treatment compared with the controls, and it was suppressed with sub-depressor-dose IRB treatment, whereas the expression of MCP-1 in the vascular wall of the Tlr4$^{lps\text{-}d}$ mice was unaffected by AngII or IRB. NE treatment resulted in only slight increases in the expression of MCP-1 in the vascular wall compared with controls in both the WT and Tlr4$^{lps\text{-}d}$ mice.

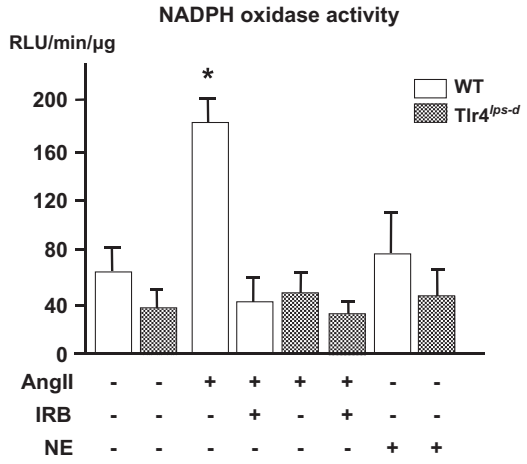

Figure 2 Activity of NADPH oxidase in the aorta. Results of a quantitative analysis of the activity of NADPH oxidase in the aorta. Bars indicate s.e. Experiments, $n=3$. $^*P<0.05$ *vs.* the other groups. AngII, angiotensin II; IRB, irbesartan; NE, norepinephrine; Tlr4$^{lps\text{-}d}$, TLR4 deficient mice; WT, wild-type.

TLR4 deficiency enhances AngII-induced upregulation of ecSOD in the aorta

In the WT mice, expression of ecSOD in the aorta was significantly increased (by threefold) with AngII treatment than in the controls, and it was sevenfold higher in the Tlr4$^{lps\text{-}d}$ mice than in the controls. These results were not affected by the presence of a sub-depressor dose of the AT1 receptor antagonist IRB, whereas its expression was inhibited by a high dose of IRB (Figure 4a). AngII also augmented the activity of ecSOD in the WT mouse aorta by 10-fold compared with that in the control mice; the ecSOD activity induced a much greater increase in these values in the Tlr4$^{lps\text{-}d}$ mice (Figure 4b). The ecSOD activity was inhibited by a high dose but not a sub-depressor dose of IRB. In contrast, NE treatment did not show any significant effects on the expression or activity of ecSOD in the aorta in either the WT or Tlr4$^{lps\text{-}d}$ mice (Figures 4a and b). Furthermore, the expression of Cu/ZnSOD in the aorta was unchanged by these drug treatments in both the WT and Tlr4$^{lps\text{-}d}$ mice (Figure 4c).

The wall-to-lumen ratio was positively correlated with NADPH oxidase activity, $\cdot O_2^-$ content and MCP-1 but not with ecSOD activity

We evaluated the correlations of the wall-to-lumen ratio with NADPH oxidase activity, $\cdot O_2^-$ content, MCP-1 and ecSOD activity in the aorta. Interestingly, quantitative analysis showed significant, positive correlations of the wall-to-lumen ratio with NADPH oxidase activity, $\cdot O_2^-$ content and MCP-1 expression in the aorta (Figures 5a–c) but no correlation between the wall-to-lumen ratio and ecSOD activity (Figure 5d).

DISCUSSION

In the present study, we showed that TLR4 deficiency prevented AngII-induced vascular remodeling without affecting blood pressure,

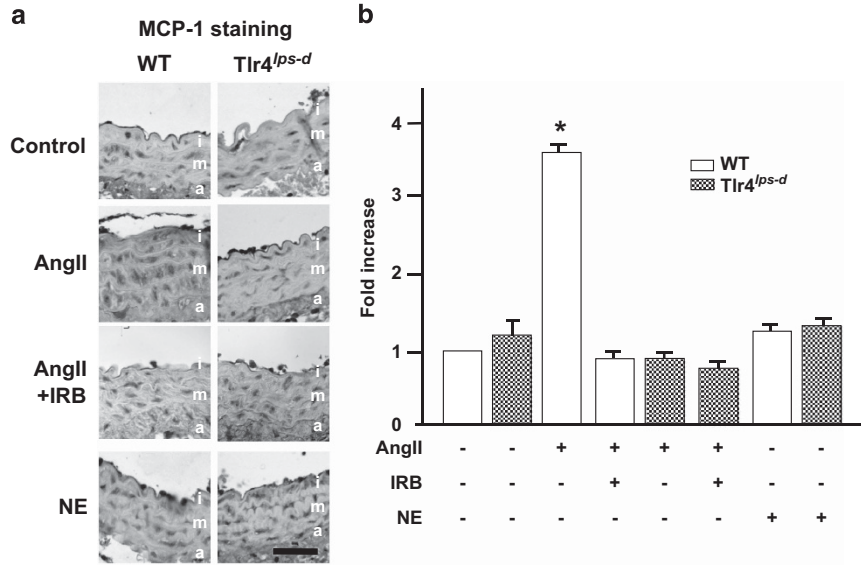

Figure 3 Expression of MCP-1 in the aorta. (**a**) Immunohistochemical staining of MCP-1 in the aorta. MCP-1 was stained brown in the cytoplasms of both endothelial and medial cells in the aorta. Bar, 50 μm. I, intima; m, media; a, adventitia. (**b**) Results of quantitative analysis of the ratio of the expression of MCP-1 to the area of tunica media in the aorta. Bars indicate s.e. Experiments, $n=3$. $^*P<0.05$ *vs.* the other groups. AngII, angiotensin II; IRB, irbesartan; NE, norepinephrine; Tlr4$^{lps\text{-}d}$, TLR4-deficient mice; WT, wild-type. A full color version of this figure is available at *Hypertension Research* online.

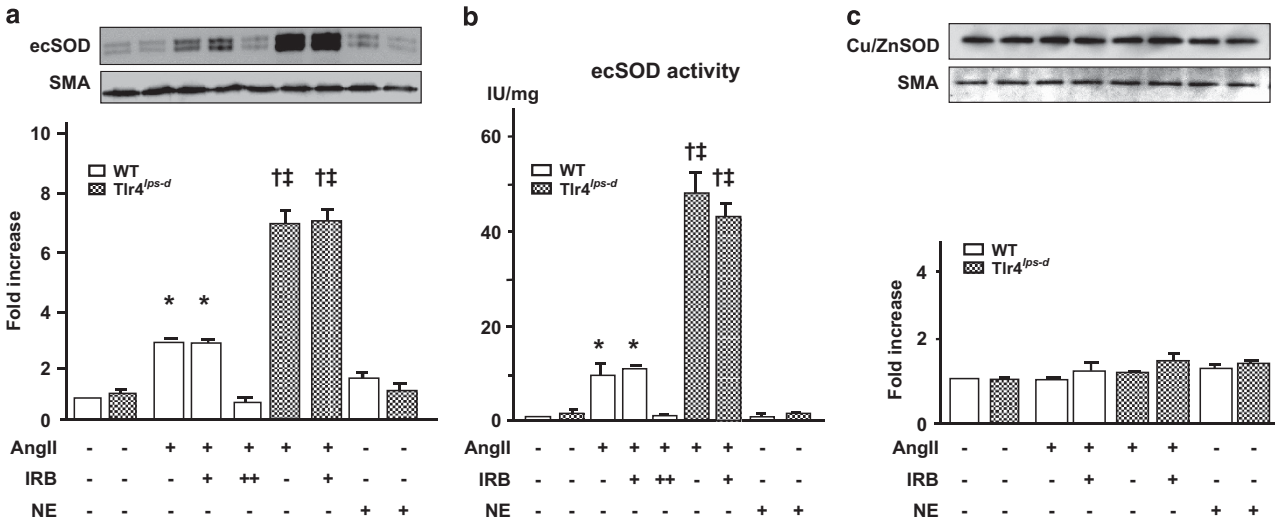

Figure 4 Expression of ecSOD and Cu/ZnSOD and the activity of ecSOD in the aorta. (**a**) Western blot analysis of the expression of ecSOD in the aorta. Anti-ecSOD antibody specifically recognized mouse ecSOD by immunoblotting (doublet 31/35 kDa). $^{*}P<0.05$ *vs.* control WT mice, $^{\dagger}P<0.05$ *vs.* control Tlr4$^{lps\text{-}d}$ mice, $^{\ddagger}P<0.05$ *vs.* AngII-treated WT mice. Bars indicate s.e. IRB doses used in the experiment; +: 1.1 mg kg^{-1} per day, ++: 21.2 mg kg^{-1} per day, respectively. (**b**) The activity of ecSOD in the aorta. $^{*}P<0.05$ *vs.* control WT mice, $^{\dagger}P<0.05$ *vs.* control Tlr4$^{lps\text{-}d}$ mice, $^{\ddagger}P<0.05$ *vs.* AngII-treated WT mice. Bars indicate s.e. IRB doses used in the experiment; +: 1.1 mg kg^{-1} per day, ++: 21.2 mg kg^{-1} per day, respectively. (**c**) Western blot analysis of the expression of Cu/ZnSOD in the aorta. Bars indicate s.e. Experiments, $n=3$. AngII, angiotensin II; IRB, irbesartan; NE, norepinephrine; SMA, α-smooth muscle actin; Tlr4$^{lps\text{-}d}$, TLR4-deficient mice; WT, wild-type.

which was associated with a decrease in vascular NADPH oxidase activity, as well as a decrease in ROS levels. Similar effects were observed in WT mice treated with sub-depressor dose of the AT1 receptor antagonist IRB. We also found that AngII-induced vascular remodeling was positively correlated with NADPH oxidase activity and ROS levels. Notably, chronic NE infusion, which elevates blood pressure without increasing ROS production, did not induce significant vascular remodeling in WT mice, suggesting that ROS elevation is required for accelerating vascular remodeling but not for hypertensive effects in this model. There have been several reports to support our current finding that TLR4 deficiency inhibited AngII-induced NADPH oxidase-derived ROS. Park *et al.*[19] reported that direct interaction of TLR4 with NADPH oxidase was involved in LPS-induced production of ROS. De Batista *et al.*[31] also demonstrated, using cultured vascular smooth muscle cells derived from spontaneously hypertensive rats, that AngII increased TLR4 mRNA levels and that an AT1 receptor blocker reduced the increase in TLR4 mRNA, whereas a TLR4 inhibitor also diminished AngII-induced increases in NADPH oxidase activity and $\cdot O_2^-$ production, migration and proliferation in rat vascular smooth muscle cells. Furthermore, the TLR4/MyD88 pathway was believed to be involved in AngII-promoted cell apoptosis, which was related to TLR4/MyD88-mediated oxidative stress.[32] Taken together, our findings suggested that TLR4 plays a pivotal role in regulating AngII-induced vascular ROS by activating NADPH oxidase, which facilitates the progression of vascular remodeling.

TLR4 has been shown to be linked to NF-κB activation pathways.[33–35] It has also been suggested that in vascular smooth muscle cells of atherosclerotic arteries, proinflammatory cytokines and oxidative stress might contribute to enhanced expression of TLR4[36] and MCP-1,[37] which depend on AT1 receptors, and the proinflammatory effect of AngII was involved in the AT1/TLR4/NF-κB signaling pathway.[38] Taken together with our study and with previous results,[36–39] it is suggested that the AT1/TLR4/NF-κB pathway might be crucial and essential for AngII-induced NADPH oxidase-derived ROS production, inflammation and vascular remodeling.

Our study demonstrated that TLR4 was not directly involved in blood pressure control, and AngII-induced hypertension was not dependent on the ROS level in our study model. There have been conflicting results regarding the role of AngII-generated ROS actions in various vascular consequences, including hypertension.[1,2] For example, inhibition of NADPH oxidase activity by genetic deletion of a component (Nox1 or Nox2), which is associated with a decrease in ROS levels, prevented the development of hypertension and cardiac hypertrophy in mice with chronic AngII infusion but not in mice crossbred with transgenic mice expressing human renin, which had high circulating AngII.[1] The reasons for these discrepancies are unclear, but they might be related to differences in genetic background (C57Bl/6 *vs.* Balb/c) and the duration of exposure to either increased ROS or AngII, that is, lifetime (genetic model) *vs.* several weeks (infusion model).

In mammals, three isoforms of SOD exist: cytoplasmic Cu/ZnSOD, mitochondrial MnSOD and ecSOD.[4] ecSOD is a primary secretory copper-containing antioxidant enzyme expressed highly in blood vessels and secreted into the extracellular space, and it regulates AngII-induced hypertension by modulating levels of extracellular $\cdot O_2^-$.[8,40,41] Intriguingly, we found that Tlr4$^{lps\text{-}d}$ mice showed enhanced activity and expression of ecSOD induced by AngII, without affecting Cu/ZnSOD expression. A high dose but not a sub-depressor dose of the AT1 receptor antagonist IRB inhibited the activity of ecSOD, whereas a sub-depressor dose of IRB inhibited AngII-induced ROS levels and NADPH oxidase activity. These findings might suggest that Ang II-induced effects on NADPH oxidase and ecSOD are mediated by distinct AT1 receptor subtypes.[42] Consistent with these data, it was reported that Ang II-induced increases in ecSOD expression were dependent on AT1 receptor.[5] Our results suggested that activation of TLR4, which can promote ROS generation and inflammation followed by vascular remodeling, negatively regulates AT1 receptor-mediated upregulation of ecSOD in the vasculature.

The precise mechanisms involved in AngII-induced upregulation or TLR4 modulation of ecSOD in this study remain unknown. It has

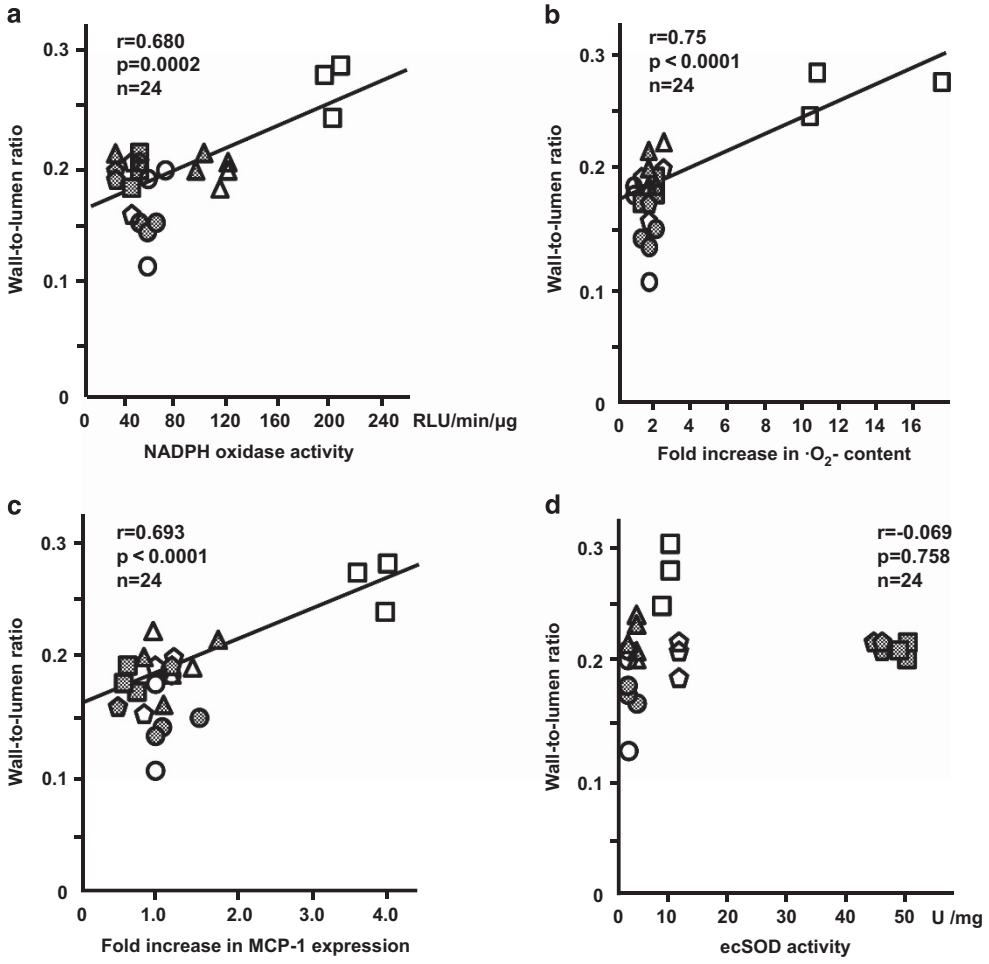

Figure 5 Relationship between the wall-to-lumen ratio and NADPH oxidase activity (**a**), superoxide ($\cdot O_2^-$) content (**b**), MCP-1 (**c**) and ecSOD activity (**d**) in the aorta. Each point represents a different mouse. AngII, angiotensin II; IRB, irbesartan; NE, norepinephrine; $Tlr4^{lps\text{-}d}$, TLR4-deficient mice; WT, wild-type. WT control mice (◯), AngII-treated WT mice (□), AngII+IRB-treated WT mice (⬠), NE-treated WT mice (△), $Tlr4^{lps\text{-}d}$ control mice (◍), AngII-treated $Tlr4^{lps\text{-}d}$ mice (▩), AngII+IRB-treated $Tlr4^{lps\text{-}d}$ mice (◈) and NE-treated $Tlr4^{lps\text{-}d}$ mice (◬).

been reported that AngII upregulates ecSOD expression in smooth muscle cells independently of NADPH oxidase-derived $\cdot O_2^-$ production,[5,43,44] and endogenous TLR ligands might be released during tissue damage, driving inflammation in the absence of infection,[12,13] thus suggesting a potential role for these endogenous ligands that involves the TLR4 receptor in arterial remodeling. Furthermore, it was recently reported that the copper transporter Menkes ATPase played an important role in modulating AngII-induced hypertension and endothelial function by regulating ecSOD activity and vascular $\cdot O_2^-$ production.[30] Notably, TLR4 deficiency markedly enhances the specific activity of ecSOD, as determined by the ratio of ecSOD activity to ecSOD protein in the vascular tissue of mice treated with chronic AngII infusion, suggesting that regulation of ecSOD activity by TLR4 occurs, at least in part, at the posttranslational level. Thus, it is conceivable that endogenous ligands and other enzymes, such as Menkes ATPase, might also be involved in AngII-induced upregulation of ecSOD, ROS production and vascular remodeling through the TLR signaling cascade, as shown in our study.

In conclusion, our findings provided novel information regarding one of the important mechanisms for the progression of vascular remodeling in hypertension. It was reported that AngII mediated ROS production followed by hypertension, endothelial dysfunction and atherosclerosis,[1] whereas in the presence of TLR4, modulation of the scavenging system against ROS in the vasculature might also play a crucial role on the progression of hypertensive vascular remodeling. In the present study, we clearly demonstrated that TLR4 could play a pivotal role in regulating AngII-induced ROS in the vasculature; that is, TLR4 might generate ROS more efficiently and might enhance the inflammation that leads to vascular remodeling through the activation of NADPH oxidase and MCP-1 and the inhibition of ecSOD. On the basis of our results, the inhibition of TLR4 could have additional therapeutic potential by both supporting the scavenging system against ROS and inhibiting the ROS-generating system to rescue the vascular wall from the progression of vascular remodeling in AngII-induced hypertension. Further *in vitro* studies are necessary to determine whether AngII stimulation is similarly dependent on TLR4 and ecSOD activity.

CONFLICT OF INTEREST

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