# Blockade of TLR4 Within the Paraventricular Nucleus Attenuates Blood Pressure by Regulating ROS and Inflammatory Cytokines in Prehypertensive Rats

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**BACKGROUND**

Toll-like receptor 4 (TLR4) has been implicated in the progression of cardiovascular disease, including hypertension. However, the role of TLR4 in the development of prehypertension is uncertain.

**METHODS**

Prehypertensive rats were treated with 8% salt for 12 weeks to induce prehypertension. These rats were then given either TAK-242 selective TLR4 blocker, or vehicle by bilateral micro-injection to the paraventricular nucleus (PVN). Blood pressure (BP) and renal sympathetic nerve activity were recorded. PVN expression of TLR4, myeloid differentiation factor 88 (Myd88), nuclear factor-kappa B (NF-κB) p65, proinflammation cytokines (PICs), interleukin (IL)-1β, IL-6, tumor necrosis factor-alpha (TNF-α), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 (NOX2), NADPH oxidase 4 (NOX4), Cu/Zn superoxide dismutase (SOD) level, tyrosine hydroxylase, and 67 kDa isoform of glutamate decarboxylase (GAD67) were tested to determine the influence of TLR4 blockade.

**RESULTS**

TLR4 expression increased significantly in the PVN of high-salt groups with a corresponding increase in reactive oxygen species (ROS) and PICs. TLR4 blockade significantly reduced the signaling molecules downstream TLR4 and the expression of TNF-α, IL-6, IL-1β, decreased ROS, NOX2, NOX4 level, increased Cu/Zn-SOD, re-balanced neurotransmitters, and regulated sympathetic nerve activity in the PVN of prehypertensive rats.

**CONCLUSIONS**

Salt-induced prehypertension is partly due to the upregulation of TLR4 in PVN. Blockade of TLR4 in the brain reduced salt-induced prehypertension response, possibly through downregulation of ROS and PICs expression, and the restorage of neurotransmitter balance in the PVN.

*Keywords:* blood pressure; hypertension; hypothalamic paraventricular nucleus; inflammatory cytokines; neurotransmitters; prehypertension; ROS; TLR4.

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Hypertension is the most common cardiovascular risk factor.[1] Chronic inflammation, oxidative stress, and sympathetic tone are crucial factors for the development and the subsequent complications of hypertension.[2] Toll-like receptor 4 (TLR4) has a vital function in inflammatory and oxidative stress processes, which affects the cardiovascular system, including hypertension.[3–5] However, the role of TLR4 mechanism associated with prehypertension is uncertain. TLR4 is an important receptor of the innate immune system that responds to various infectious agents and stressors.[6,7] TLR4 signal activation is known by an increase in proinflammation cytokines (PICs) expression through nuclear factor-kappa B (NF-κB) activation, and results in an inflammatory process characterized mainly by the production of PICs, such as interleukin (IL)-1β , IL-6, and tumor necrosis factor-alpha (TNF-α).[8] There is unequivocal fact that the central nervous system plays an important role in hypertension in parallel with inflammation.[9–11] Increased levels of PICs within the paraventricular nucleus (PVN) are crucial in the progression of hypertension,[12] and blockade of TLR4 in PVN can slow down the development of hypertension administered by angiotensin II.[13]

Hypertension has been linked to the generation of oxidative stress resulting from chronic inflammation.[14] Previous studies have shown that generation of oxidative stress

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involved in TLR-dependent signaling pathway in cells from immune and nonimmune origins are produced mainly through the stimulation of several NADPH oxidase (NOX) isoforms.[15,16] NOX is the main enzyme responsible for reactive oxygen species (ROS). It is a multienzymatic complex formed by gp91phox (NOX2) and its homologous NOX1, NOX4, rac, p22phox, p47phox, and p67phox. In our previous work, we found an increase in the expression of the catalytic NADPH oxidase subunit NOX2 and NOX4 in the PVN of various hypertensive rats.[17–19] The stimulation of NOX enzymes serves as an important process associated with TLRs' generation of secondary ROS, which leading to a series of continuous amplification of the original inflammatory response. This process "TLR-radical cycle" may maintain the chronic inflammation in hypertension.[20]

The PVN is a major region regulating nervous signals for maintaining resting blood pressure (BP) and sympathetic tone, and produces excitatory and inhibitory neurotransmitters, which play their roles to coordinate autonomic and neuroendocrine homeostasis. A growing body of evidence indicates that the enhanced sympathoexcitation during hypertension is due to an increase in excitatory adrenergic and glutamatergic activities and a decrease in GABAergic activity in PVN. Our lab found that rats suffered from heart failure had increased neuronal excitation accompanied by increased level of tyrosine hydroxylase (TH) and decreased level of gamma-aminobutyric acid (GABA) in the PVN.[21] A previous study from our lab also demonstrated that rats treated with high-salt (HS) diet for 15 weeks increased in BP and renal sympathetic nerve excitability (RSNA), while oral administration of antioxidant and free-radical scavenger CoQ10 attenuated the HS-induced hypertension *via* regulating ROS, PICs, NE, and GABA in PVN.[22]

Accordingly, we hypothesized that HS intake would lead to TLR4 activation and induce the production of ROS, PICs, amplify the inflammatory reaction, imbalanced excitatory and inhibitory neurotransmitters, increase in sympathetic excitability, and elevated BP as part of the pathogenesis of hypertension. In this study, we determined whether blockade of TLR4 in the PVN of prehypertensive rats can attenuate hypertensive responses and sympathoexcitation *via* inhibition of ROS, PICs, and re-balancing the neurotransmitters.

## MATERIALS AND METHODS

### Animals

Male Sprague-Dawley rats were used in this study. The rats were kept and maintained at temperatures of 20–23°C under controlled 12h/12h dark/light cycle. The experimental procedures in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals were duly followed. Approval for our study was obtained from the Xi'an Jiaotong University Committee for Animal Research.

### General experimental protocol

NaCl of 0.3% and 8% was administered to normal salt (NS) group and the HS group over a period of 3 months respectively. The rats were put into 4 groups: (i) NS + vehicle; (ii) NS + TAK-242; (iii) HS + vehicle; and (iv) HS + TAK-242. Bilateral cannulae were implanted into the PVN of prehypertensive rats for infusion of TAK-242 (10 μg/h), a selective TLR4 blocker, or artificial cerebrospinal fluid (vehicle). We refer to the relevant research,[23,24] and the amount of the TAK-242 used was based on a study which employed 2, 10, and 50 μg/h dose. The high dose caused mortality while the 10 μg/h produced optimal response but the inhibition effect of the low dose was incomplete. After 4-week drug intervention, the rats were administered an anesthesia of ketamine

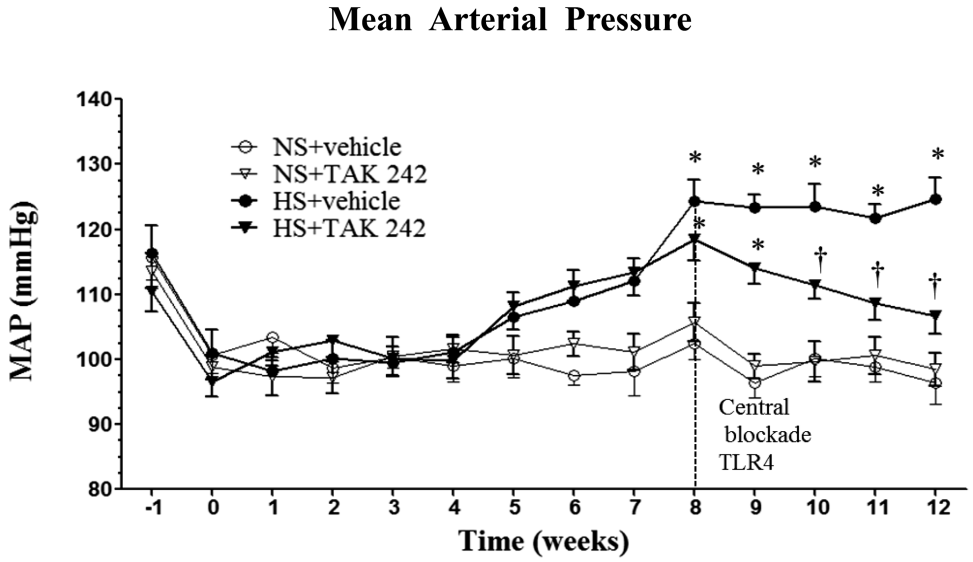


**Figure 1.** Prehypertensive rats were induced with diet containing 8% NaCl. Two months later, the blood pressure increased significantly in HS group compared to the NS group. Chronic PVN infusion of TAK-242, a selective TLR4 antagonist, for 4 weeks reduced the blood pressure of prehypertensive rats, as compared to control group. $N = 6–8$ per group. Data are presented by means ± SEM. $^{*}P < 0.05$ vs. control (NS + vehicle or NS + TAK-242), $^{\dagger}P < 0.05$ (HS + TAK-242 vs. HS + vehicle). Abbreviations: HS, high salt; NS, normal salt; PVN, paraventricular nucleus; TLR4, Toll-like receptor 4.

(80 mg/kg) and xylazine (10 mg/kg) mixture (ip); following this brains were removed and immediately frozen on dry ice.

### Tissue microdissection

Brain was sectioned serially in 300 μm increments from the bregma to lambda, both sides of the PVN tissues were isolated by the use of a punch-out technique with a cryostat, as previously described.[25,26] PVN tissue was stored at −80°C until analyzed for enzyme-linked immunosorbent assay (ELISA) or western blotting.

### The application of chronic infusion of TAK-242 in PVN

A 28-day miniosmotic pump (infusion rate 0.25 μl/h; Alzet, model 2004, Durect Corporation, Cupertino, CA) was connected to the infusion cannula through a catheter tube to deliver TAK-242 or vehicle in the PVN, as described previously.[27]

**Table 1.** Significant changes in body weight, blood pressure, and heart rate were observed on the 12th week of HS diet

| Parameters | NS + vehicle | NS + vehicle | HS + vehicle | HS + TAK-242 |
|---|---|---|---|---|
| Body weight, g | 355 ± 25 | 351 ± 27 | 345 ± 16 | 346 ± 19 |
| MAP, mm Hg | 96 ± 9 | 98 ± 7 | 125 ± 8* | 107 ± 7† |
| HR, bpm | 353 ± 10 | 358 ± 12 | 385 ± 12* | 356 ± 10† |

*N* = 6–8 per group. Data are presented by means ± SEM. **P* < 0.05 vs. control (NS + vehicle or NS + TAK-242), †*P* < 0.05, HS + TAK-242 vs. HS + vehicle. Abbreviations: HR, heart rate; HS, high salt; MAP, mean arterial pressure; NS, normal salt.

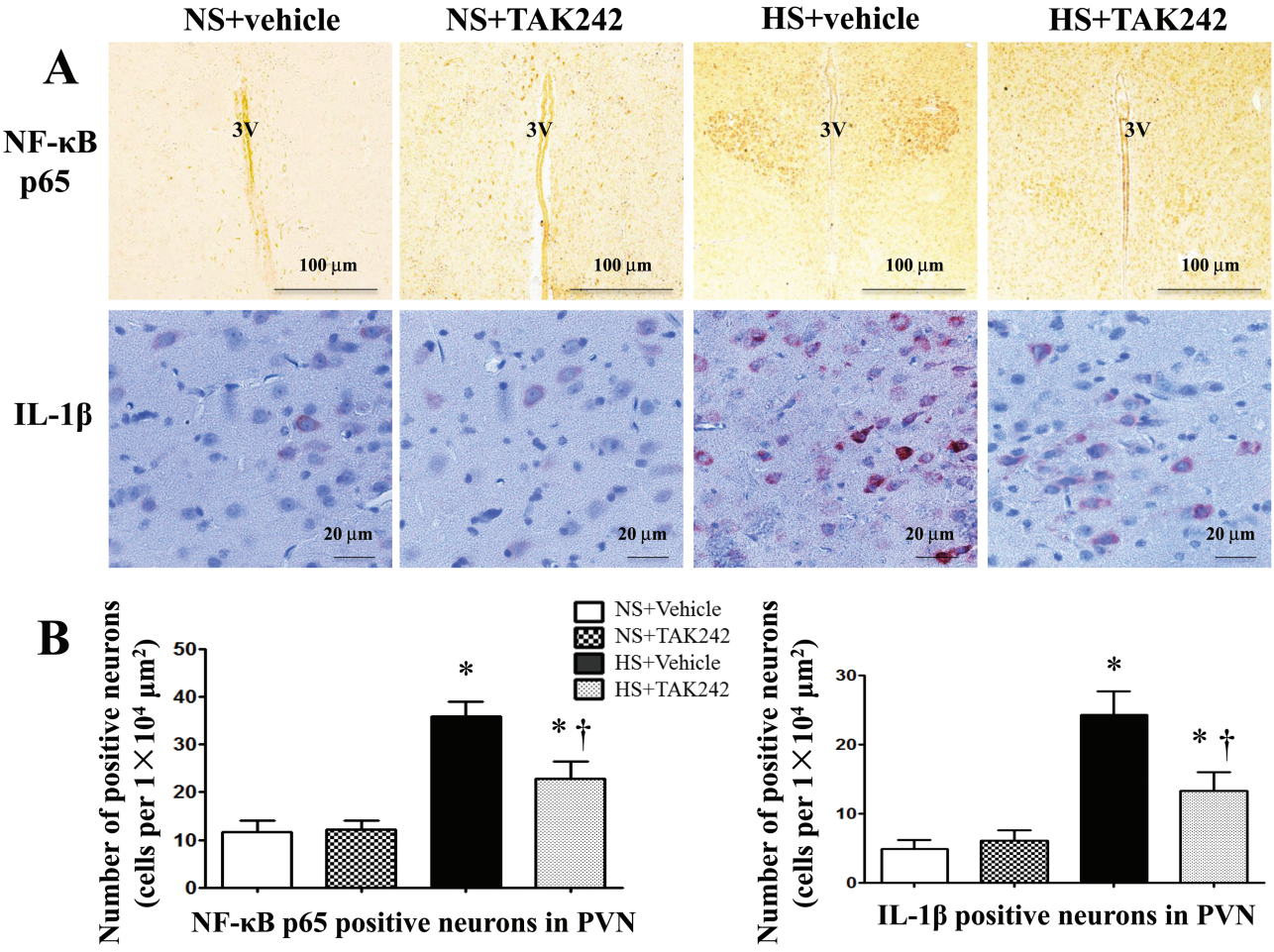


**Figure 2.** Chronic PVN infusion of TAK-242 reduced protein expression of the molecules in TLR4 pathway in HS rats. (**A**) A representative immunohistochemisty image of NF-κB p65 and IL-1β (scale bar 100 μm and 20 μm) showing the changes of NF-κB p65 and IL-1β expression after central blockade TLR4. (**B**) The numbers of positive neurons of NF-κB p65 and IL-1β in various groups. *N* = 6–8 per group. Data are presented by means ± SEM. **P* < 0.05 vs. control (NS + vehicle or NS + TAK-242), †*P* < 0.05, HS + TAK-242 vs. HS + vehicle, 3V, third ventricle. Abbreviations: HS, high salt; IL, interleukin; NF-κB, nuclear factor-kappa B; NS, normal salt; PVN, paraventricular nucleus; TLR4, Toll-like receptor 4.

### BP measurements

The noninvasive computerized tail-cuff system (NIBP, ADInstruments, Australia) was applied for measuring the tail artery BP in conscious rats, as described previously.[28] The rats were allowed to acclimatize by measuring the daily BP for at least 7 days, to reduce stress induced and prevent fluctuations in BP. For each rat, the mean arterial BP was determined and recorded on daily basis.

### Recording sympathetic activity

The rats were administered an anesthesia of ketamine (80 mg/kg) and xylazine (10 mg/kg) mixture (ip), the left renal nerves were isolated *via* retroperitoneal laparotomy under an anatomic microscope, connect the renal nerve to the recording system. Maximum RSNA was detected using an intravenous bolus administration of sodium nitroprusside (10 mg). The recordings of rectified and integrated RSNA were analyzed using methods described as previously.[26,29]

### Immunohistochemistry staining

The protocol for immune staining was done as detailed as in our previous reports.[27,30] Brains were removed, fixed in 4% buffered-paraformaldehyde for 24 hours. Paraffin section was prepared in conventional method, serials 5 μm sections of brain were cut on a semiautomatic microtome. The primary antibody was diluted in 0.1 phosphate buffer with 5% normal goat serum incubated overnight at 4°C. The following antibodies were immunohistochemically and immunofluorescently determined: NF-κB (1:200, Santa Cruz, CA), IL-1β (1:100, Santa Cruz), NOX2 (1:50, Santa Cruz), NOX4 (1:100, Santa Cruz), TH (1:200, Santa Cruz), 67 kDa isoform of glutamate decarboxylase (GAD67) (1:200, Santa Cruz). The images were taken by a laser-scanning confocal imaging system (Leica SP5) and Olympus microscope (IX71; Olympus). The Image-Pro Plus software was applied in the analysis of the integral optical density and fluorescence intensity.

### Measurement of ROS in PVN

ROS in the PVN was evaluated by the oxidative fluorescence dye dihydroethidium. Brain sections were incubated for 10 minutes with dihydroethidium (1 μmol/l, Sigma) at 37°C in the dark, as previously described.[31] The oxidative fluorescence intensity was detected at 585 nm wave length by a laser-scanning confocal imaging system (Leica SP5).

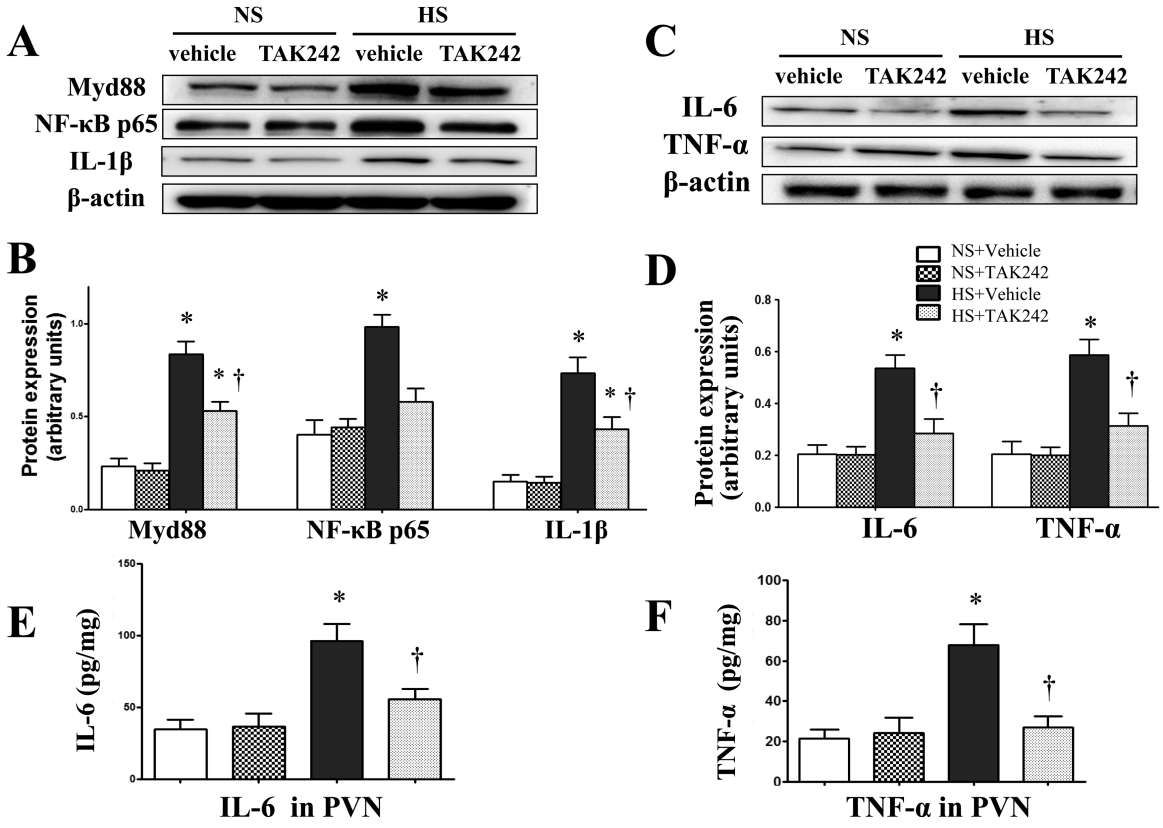


**Figure 3.** Central blockade of TLR4 suppresses TLR4 pathway, IL-6, and TNF-α expression in prehypertensive rats. (**A**) A representative immunoblot of Myd88/NF-κB p65/IL-1β. (**B**) Densitometric analysis of the expression of Myd88/NF-κB p65/IL-1β in various groups, $n = 3$ per group. (**C**) A representative immunoblot of IL-6 and TNF-α expression in PVN. (**D**) Analysis of the expression of IL-6 and TNF-α in PVN in different groups, $n = 3$ per group. (**E**) ELISA detected the IL-6 expression in the different groups, $n = 6–8$ per group. (**F**) ELISA detected the TNF-α expression in the various groups, $n = 6–8$ per group. Data are presented by means ± SEM. $^{*}P < 0.05$ vs. control (NS + vehicle or NS + TAK-242), $^{\dagger}P < 0.05$, HS + TAK-242 vs. HS + vehicle. Abbreviations: ELISA, enzyme-linked immunosorbent assay; IL, interleukin; Myd88, myeloid differentiation factor 88; NF-κB, nuclear factor-kappa B; NS, normal salt; PVN, paraventricular nucleus; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-α.

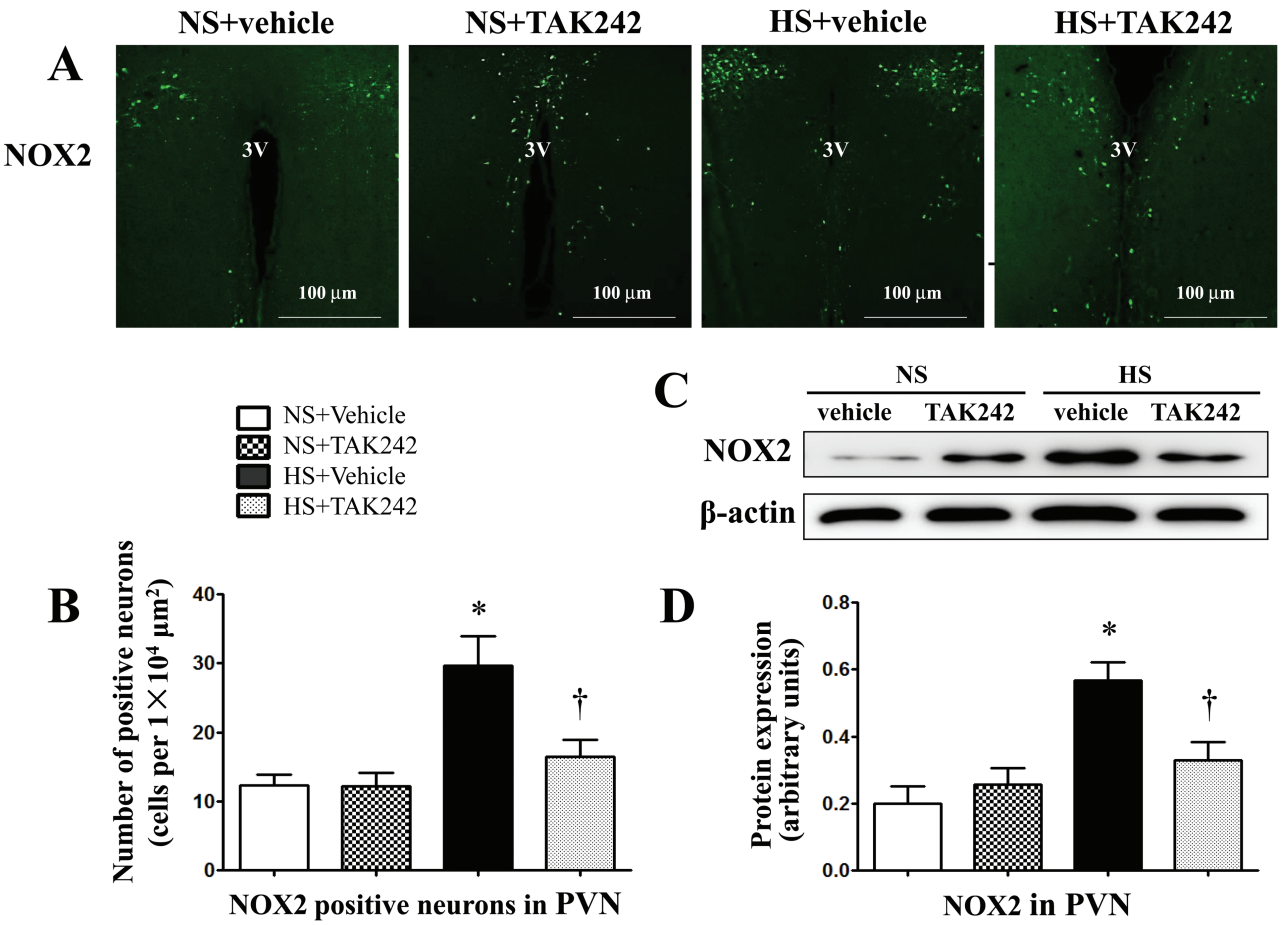


**Figure 4.** Central blockade of TLR4 inhibited NOX2 expression in prehypertensive rats. (**A**) A representative immunofluorescence image of NOX2, scale bar 100 μm. (**B**) The numbers of neurons positive for NOX2 in various groups; $n = 6–8$ per group. (**C**) A representative immunoblot of NOX2; (**D**) Densitometric analysis for the expression of NOX2 in various groups, $n = 3$ per group. Data are presented by means ± SEM. *$P < 0.05$ vs. control (NS + vehicle or NS + TAK-242), †$P < 0.05$, HS + TAK-242 vs. HS + vehicle, 3V, third ventricle. Abbreviations: HS, high salt; NOX2, NADPH oxidase 4; NS, normal salt; TLR4, Toll-like receptor 4.

Coronal sections were photographed and fluorescence intensity was quantified using Image-Pro Plus software.

### Western blotting

Western blotting analysis was performed in the same manner as previously described.[13] Protein extracts (40 μg) were combined with an equal volume of 5× loading buffer, boiled for 5 minutes and electrophoresed on 10–15% sodium dodecyl sulfate-polyacrylamide gels. The proteins were then electroblotted onto polyvinylidene fluoride membranes (Immobilon-P, Millipore). Nonspecific binding was blocked by incubating the membranes in 1% casein in phosphate-buffered saline-Tween for 1 h at room temperature. Blots were then incubated overnight at 4°C with the primary antibodies. The primary antibodies as the following: myeloid differentiation factor 88 (Myd88) (1:500, Santa Cruz), NF-κB (1:1000, Santa Cruz), IL-1β (1:500, Santa Cruz), NOX2 (1:500, Santa Cruz), NOX4 (1:500, Santa Cruz), Cu/Zn superoxide dismutase (SOD) (1:500, Santa Cruz), TH (1:2000, Santa Cruz), GAD67 (1:1000, Santa Cruz). The β-actin antibody was used as an internal standard and band densities were analyzed with NIH Image J software.

### Cytokines measure

Commercially available rat ELISA kits were used to quantify NE (Abnova, Taiwan), TNF-α, and IL-6 (Invitrogen Corporation, CA) according to the manufacturer's instructions. Solid phase of the microtiter plate was bound with the sample 10 μl whose concentration was determined using a microplate reader at 450 nm. All the samples were assayed in duplicates.

### Data analysis

Data analyses were conducted by either a 1-way or 2-way analysis of variance followed by a *post hoc* Bonferroni test. Data for BP were analyzed using repeated measures analysis of variance. Data were recorded as mean ± SEM. All data were deemed as statistically significant if probability value of $P < 0.05$.

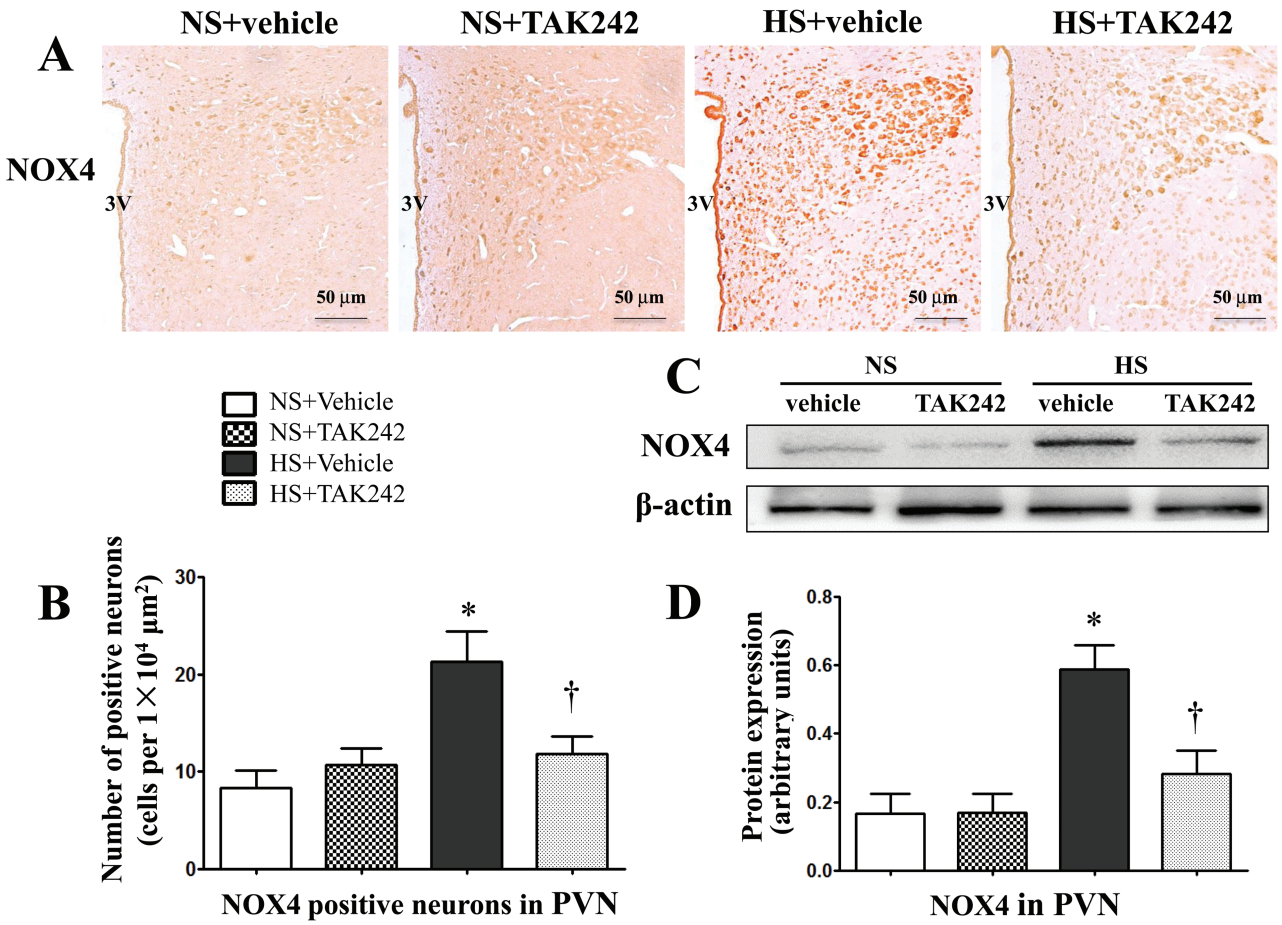


**Figure 5.** Central blockade of TLR4 inhibited NOX4 expression in prehypertensive rats. (**A**) A representative immunohistochemisty image of NOX4 showing the changes of NOX4 after central blockade of TLR4, scale bar 50 μm. (**B**) The numbers of neurons positive for NOX4 in various groups; $n = 6–8$ per group. (**C**) A representative immunoblot of NOX4. (**D**) Densitometric analysis for the expression of NOX4 in various groups, $n = 3$ per group. Data are presented by means ± SEM. *$P < 0.05$ vs. control (NS + vehicle or NS + TAK-242), †$P < 0.05$, HS + TAK-242 vs. HS + vehicle, 3V, third ventricle. Abbreviations: HS, high salt; NOX2, NADPH oxidase 4; NS, normal salt; TLR4, Toll-like receptor 4.

## RESULTS

### The expression of TLR4 in the rats with HS diet after 2 months

TLR4 expression in the PVN of rats with HS diet increased significantly in the second and third month of HS group compare to the control group, as shown in Supplementary Figure S1B and C. Most interestingly, the TLR4 expression had no difference between the HS diet for 2 and 3 months.

### The role of TLR4 on BP of salt-induced prehypertensive rats

Blood pressure in HS rats was increased significantly compare to the NS group since the eighth week of HS diet, which is parallel to the increase of TLR4 in PVN. Chronic PVN infusion of TAK-242, a selective TLR4 blocker, with a dosage of 10 μg/h for 4 weeks, led to mean arterial pressure drop significantly in the group with TAK-242 treatment compare to control group (Figure 1 and Table 1).

### Central blockade TLR4 suppressed the Myd88/NF-κB p65/IL-1β expression in the PVN of prehypertensive rats

To determine whether the suppression of TLR4 in PVN reduces inflammatory response, we detected the Myd88/NF-κB p65/IL-1β expression in the by immunohistochemistry. Chronic infusion of TAK-242 in PVN reduced NF-κB p65, IL-1β expression in the prehypertensive rats (Figure 2). After 4-week treatment with TAK-242, decreased TLR4 pathway expression was found, which is consistent with the result of Dange.[13,32] Myd88/NF-κB p65/IL-1β expressions were further analyzed using the western blotting technique (Figure 3A and B), and the results showed that the expression of all these 3 molecules decreased. Meanwhile, TAK-242 infusion did not affect the Myd88/NF-κB p65/IL-1β expression in the NS rats.

### Central blockade of TLR4 reduced level of TNF-α and IL-6 in the PVN of HS rats

To determine the role of TLR4 during inflammatory response, TNF-α and IL-6 expression in PVN of all

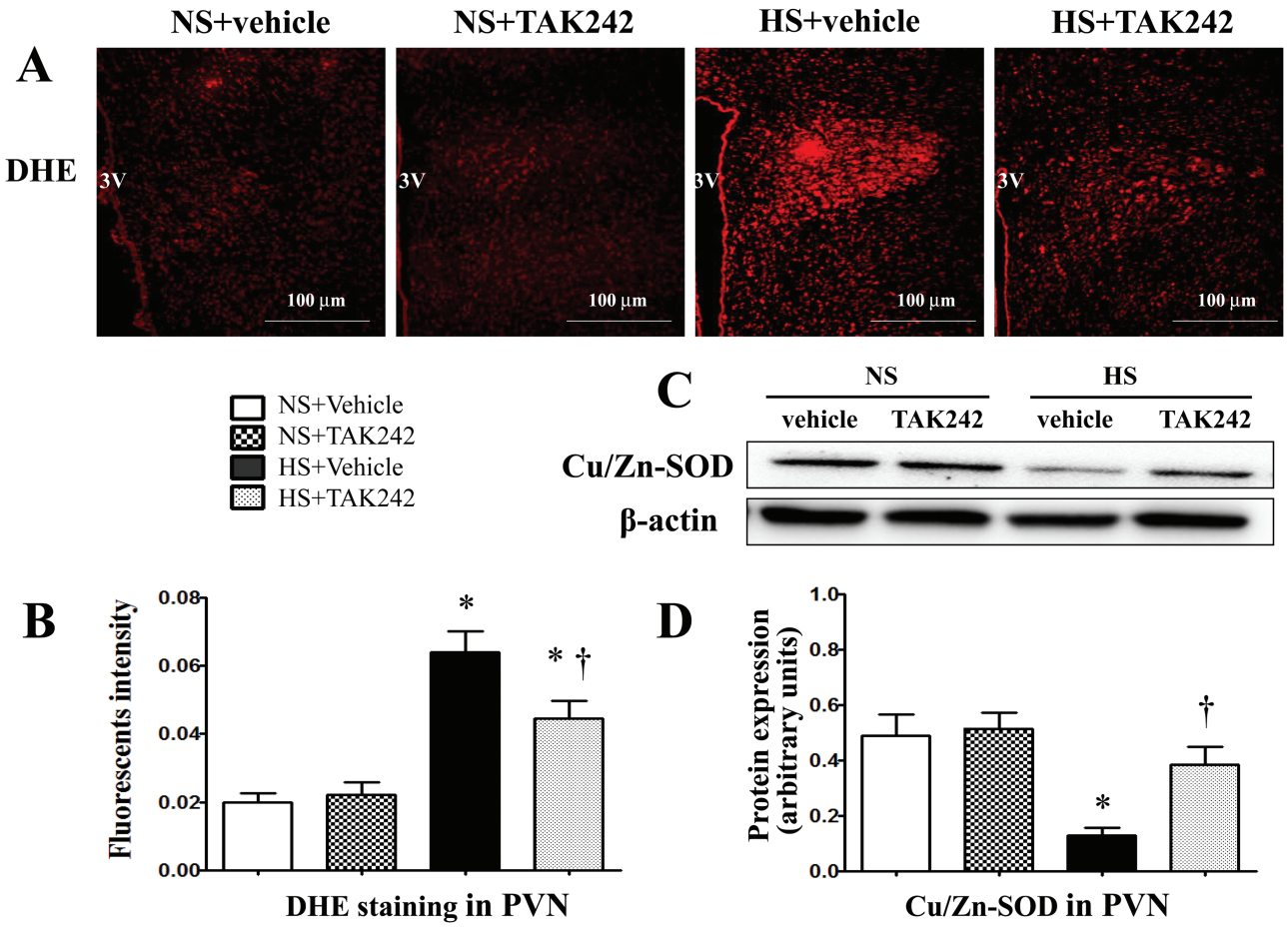


**Figure 6.** Central blockade of TLR4 reduced PVN superoxide in prehypertensive rats. (**A**) Superoxide was measured by fluorescent-labeled DHE staining, scale bar 100 μm. (**B**) Immunofluorescent intensity of DHE in various groups; $n = 6–8$ per group. (**C**) A representative immunoblot of Cu/Zn-SOD; (**D**) Densitometric analysis for the expression of Cu/Zn-SOD in various groups, $n = 3$ per group. Data are presented by means ± SEM. $^{*}P < 0.05$ vs. control (NS + vehicle or NS + TAK-242), $^{\dagger}P < 0.05$, HS + TAK-242 vs. HS + vehicle, 3V, third ventricle. Abbreviations: DHE, dihydroethidium; HS, high salt; NOX2, NADPH oxidase 4; NS, normal salt; PVN, paraventricular nucleus; SOD, superoxide dismutase; TLR4, Toll-like receptor 4.

groups were examined. ELISA and western blotting analysis showed that HS rats exhibited significantly higher expression of TNF-α and IL-6 in the PVN compared to control group (Figure 3C–F). The upregulation of TNF-α and IL-6 were significantly reduced by central blockade of TLR4. Moreover, TNF-α and IL-6 expression in PVN of NS groups did not change after treatment with TAK-242.

### Central blockade of TLR4 reduced level of NOX2 and NOX4 in HS rats

To determine whether blockade of TLR4 in PVN regulates subunit of NADPH oxidase, expression of NOX2 and NOX4 were examined by immunohistochemistry staining and western blotting (Figures 4 and 5). Compared with the control group, HS + vehicle group showed significant increase in NOX2 and NOX4 expression. Blockade of TLR4 in PVN decreased the level of NOX2 and NOX4 in HS rats. These observations revealed that central blockade of TLR4 will reduce the level of NOX2 and NOX4 expression in salt-induced prehypertensive rats.

### Central blockade of TLR4 decreases superoxide and increases Cu/Zn-SOD in prehypertensive rats

Immunofluorescence and western blotting showed that PVN superoxide in prehypertensive rats increased significantly compared to the control group, as demonstrated by dihydroethidium and Cu/Zn-SOD level. Chronic infusion of TAK-242 decreased dihydroethidium and increased Cu/Zn-SOD in the PVN of HS rats (Figure 6).

### Central blockade of TLR4 regulates neurotransmitters in HS rats

To investigate the effect of TAK-242 infusion on the neurotransmitters in prehypertensive rats, we assayed the levels of plasma NE, TH, and GAD67 in PVN. The HS rats had higher NE expression compared with the control group. Chronic infusion of TAK-242 in PVN attenuated plasma NE in prehypertensive rats. Compared to NS rats, HS rats had higher expression of TH and lower GAD67 in the PVN. Blockade of TLR4 in PVN lowered TH and upregulated GAD67 expression in prehypertensive rats (Figure 7).

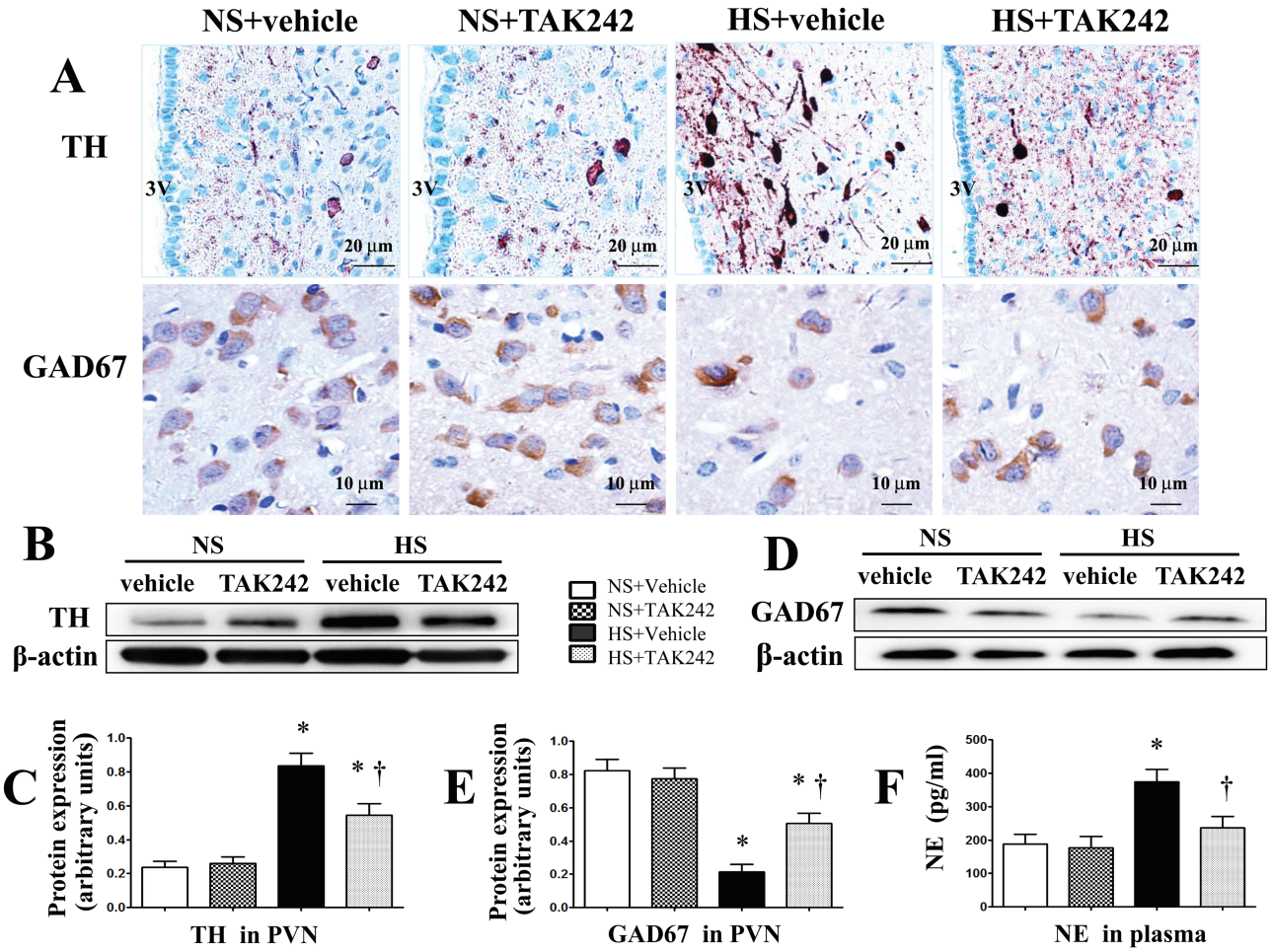


**Figure 7.** Central blockade of TLR4 reduced TH and 67 kDa isoform of GAD67 in the PVN of prehypertensive rats. (**A**) Representative immunohistochemistry images of TH and GAD67 showing the changes of TH and GAD67 after central blockade TLR4, scale bar 20 μm and 10 μm. (**B**) A representative immunoblot of TH. (**C**) Densitometric analysis for the expression of TH in various groups, $n = 3$ per group. (**D**) A representative immunoblot of GAD67. (**E**) Densitometric analysis for the expression of GAD67 in various groups, $n = 3$ per group. (**F**) ELISA detected plasma NE level in the various groups, $n = 6$–8 per group. Data are presented by means ± SEM. *$P < 0.05$ vs. control (NS + vehicle or NS + TAK-242), †$P < 0.05$, HS + TAK-242 vs. HS + vehicle, 3V, third ventricle. Abbreviations: ELISA, enzyme-linked immunosorbent assay; GAD67, 67 kDa isoform of glutamate decarboxylase; HS, high salt; NOX2, NADPH oxidase 4; NS, normal salt; PVN, paraventricular nucleus; SOD, superoxide dismutase; TH, tyrosine hydroxylase; TLR4, Toll-like receptor 4.

### Effect of TLR4 on RSNA

At the end of the experiment, prehypertensive rats exhibited higher RSNA (% of max) when compared to normotensive rats; bilateral PVN blockade of TLR4 attenuated RSNA in prehypertensive rats (Supplementary Figure S1D and E).

## DISCUSSION

Recent evidence suggests a role for TLR4 in the development of cardiovascular diseases. Eissler *et al.* showed that TLR4 in cardiac increased in L-n-nitro-L-arginine methyl ester–induced hypertension.[4] Dange *et al.* demonstrated that hypertensive rats exhibited significantly higher level of TLR4 in the PVN, while central blockade of TLR4 downregulated inflammatory molecules TNF-α and IL-1β, attenuated sympathetic activity and reduced BP.[13,32] Li *et al.* showed that AT1-R antagonist within the PVN attenuated hypertension and reduced the expression of TNF-α, IL-1β, and IL-6 *via* the TLR4/MyD88/NF-κB signaling pathway in spontaneously hypertensive rats.[33] However, the role of TLR4 mechanism associated with prehypertension is uncertain.

Our findings demonstrated that prehypertensive rats had dramatically increased expression of TLR4 compared with normotensive rats. Then, what activates TLR4? What are the possible ligands of TLR4? Researchers have reported a new connection between salt and autoimmunity as high-salt diet was shown to accelerate autoimmune activity.[34,35] Recent evidence suggests that large amounts of sodium are stored in the interstitium,[36,37] this unappreciated electrolyte accumulation induces compensatory local regulatory lymphatic clearance mechanisms and parallel responses of mononuclear phagocyte system cells and T cell. NLRP3 is another well-known intracytoplasmic pattern recognition receptor. Philipp *et al.* reported HS induced a transient increase of the NLRP3 protein level and a moderate NLRP3 inflammasome

activation.[38] So, we hypothesize that the high-salt environment can activate the immune system and TLR4, which was preliminarily confirmed in this study, this puzzle needs to be further explored and discussed. We also found that central blockade of TLR4 reduced the BP, as well as the expression of Myd88/NF-κB p65, proinflammatory cytokine IL-1β, IL-6, TNF-α. These results were associated with a reduction in ROS, re-balanced excitatory, and inhibitory neurotransmitter in prehypertensive rats. Both excitatory and inhibitory neurotransmitters have been demonstrated to contribute to sympathetic activity, including NE and GABA.[39] This is consistent with the findings that brain cytokines can induce an imbalance between excitatory and inhibitory neurotransmitters in the PVN of heart failure rats, which contributes to sympathoexcitation.[21]

Oxidative stress plays a crucial role in the progression and sustenance of inflammation, and thus supports the pathophysiology of several debilitating illnesses, such as cardiovascular diseases.[17–19] Every stage of inflammatory response as well as endogenous danger signaling molecules discharged by damaged tissues and their detection by innate immune receptors from the Toll-like (TLRs) families were influenced by oxidants. In contrast, TLR activation can also induce oxidant production.[16] TLR4 stimulation with LPS induces ROS generation and subsequently NF-κB activation, indicating direct interrelation between TLR4 and NOX4 as a critical mechanism to control TLR-dependent innate immune response.[40] In the previous work, we found NADPH oxidase subunit NOX2 and NOX4 have been implicated in the development of oxidative stress and hypertension.[17–19,41] In this study, we found that prehypertensive rats showed significant increases in NOX2, NOX4, and superoxide, associated with significant higher level of TLR4 in prehypertensive rats. Blockade of TLR4 in PVN decreased expression of NOX2, NOX4, and superoxide in salt-induced prehypertensive rats, but not in normotensive rats. These observations revealed that central blockade of TLR4 decreased NAD(P)H oxidase–derived ROS level in prehypertensive rats, possibly *via* downregulation of PICs. Excessive ROS increased sympathetic nerve excitability and elevated BP.[41] Inhibition of ROS in the PVN restores the balance of neurotransmitters, attenuating hypertensive response and sympathetic activity.[18]

PVN is a crucial central control spot for the modulation of BP and sympathetic nerve activity. The PVN sympathetic discharge depends on the equilibrium between excitatory and inhibitory neurotransmitters.[30] Recent studies have indicated that hypertension is linked with elevated levels of excitatory neurotransmitters and inhibitory GABAergic system in PVN.[27,30] GABA is the principal inhibitory neurotransmitter in the PVN, inhibition of GABA receptor in the PVN increases renal sympathetic nerve activity, indicating that a strong tonic GABA-mediated inhibition of sympathetic neuronal firing exists.[42] NE is the main excitatory neurotransmitter of neuroendocrine regulation. Our data showed that RSNA was increased in prehypertensive rats, along with increase in plasma NE and decreased in the levels of GAD67, which is the rate-limiting enzyme of the inhibitory neurotransmitter GABA. We also demonstrated that the level of neuromodulator TH was increased in rats with high-salt diet. TH is a key enzyme that converts L-tyrosine to catecholamine, epinephrine, and norepinephrine. TH might be one of the determinant factors for overall sympathetic activity, as well as the development of hypertension.[43–45] In addition, we found that central blockade of TLR4 significantly reduced RSNA, plasma NE, and neuromodulators TH, as well as increased GAD67 in PVN of prehypertensive rats. These results suggest that TLR4 activation in the PVN modulates neurotransmitters and contributes to sympathoexcitation in prehypertensive rats.

## CONCLUSIONS

In this study, we demonstrated that TLR4 expression in the PVN of prehypertensive rats were increased with corresponding elevated BP, and chronic infusion of TAK-242 in PVN blocked the activation of TLR4 downstream signal, inhibited the production of inflammatory cytokines and NAD(P)H oxidase–derived ROS, alleviated oxidative stress, and inflammatory response. Central blockade of TLR4 restored the balance of excitatory and inhibitory neurotransmitters and inhibited RSNA in prehypertensive rats. These results provide the evidence that TLR4 activation contributes to the progression of prehypertension. Blockade of TLR4 in the PVN attenuates salt-induced prehypertensive response, possibly *via* downregulation of ROS, PIC, and sympathetic activity.

## SUPPLEMENTARY DATA

Supplementary data are available at *American Journal of Hypertension* online.

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## DISCLOSURE

The authors declared no conflict of interest.

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