

Toll-like receptor 4 contributes to blood pressure regulation and vascular contraction in spontaneously hypertensive rats

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A B S T R A C T

Activation of TLRs (Toll-like receptors) induces gene expression of proteins involved in the immune system response. TLR4 has been implicated in the development and progression of CVDs (cardiovascular diseases). Innate and adaptive immunity contribute to hypertension-associated end-organ damage, although the mechanism by which this occurs remains unclear. In the present study, we hypothesize that inhibition of TLR4 decreases BP (blood pressure) and improves vascular contractility in resistance arteries from SHR (spontaneously hypertensive rats). TLR4 protein expression in mesenteric resistance arteries was higher in 15-week-old SHR than in age-matched Wistar controls or in 5-week-old SHR. To decrease the activation of TLR4, 15-week-old SHR and Wistar rats were treated with anti-TLR4 (anti-TLR4 antibody) or non-specific IgG control antibody for 15 days (1 μ g per day, intraperitoneal). Treatment with anti-TLR4 decreased MAP (mean arterial pressure) as well as TLR4 protein expression in mesenteric resistance arteries and IL-6 (interleukin 6) serum levels from SHR when compared with SHR treated with IgG. No changes in these parameters were found in treated Wistar control rats. Mesenteric resistance arteries from anti-TLR4-treated SHR exhibited decreased maximal contractile response to NA (noradrenaline) compared with IgG-treated SHR. Inhibition of COX (cyclo-oxygenase)-1 and COX-2, enzymes related to inflammatory pathways, decreased NA responses only in mesenteric resistance arteries of SHR treated with IgG. COX-2 expression and TXA₂ (thromboxane A₂) release were decreased in SHR treated with anti-TLR4 compared with IgG-treated SHR. Our results suggest that TLR4 activation contributes to increased BP, low-grade inflammation and plays a role in the augmented vascular contractility displayed by SHR.

INTRODUCTION

TLR4 (Toll-like receptor 4) is expressed on virtually all human cells and binds a wide spectrum of

exogenous [including bacterial LPS (lipopolysaccharide)] and endogenous [HSP (heat-shock protein) and AngII (angiotensin II)] ligands. TLR4 is involved in innate immune responses to various infectious agents and

Key words: cyclo-oxygenase, hypertension, inflammation, mesenteric resistance artery, Toll-like receptor 4 (TLR4).

Abbreviations: AngII, angiotensin II; BP, blood pressure; CRP, C-reactive protein; COX, cyclo-oxygenase; CVD, cardiovascular disease; HR, heart rate; HSP, heat-shock protein; 6-keto PGF_{1 α} , 6-keto prostaglandin F_{1 α} ; IL, interleukin; LPS, lipopolysaccharide; MAP, mean arterial pressure; MAPK, mitogen-activated protein kinase; NA, noradrenaline; NF- κ B, nuclear factor κ B; NS-398, N-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide; PG, prostaglandin; SC-560, 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole; SHR, spontaneously hypertensive rats; TLR, Toll-like receptor; TNF α , tumour necrosis factor α ; TXA₂, thromboxane A₂; TXB₂, thromboxane B₂; VSMC, vascular smooth muscle cell.

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stressors [1]. In the presence of a ligand, the TLR4 receptor activates intracellular pathways that culminate in NF- κ B (nuclear factor κ B) phosphorylation, resulting in an inflammatory process characterized mainly by the production of pro-inflammatory cytokines [2].

TLR4 has been implicated in mediating chronic inflammatory diseases, including CVDs (cardiovascular diseases) [3]. TLR4 up-regulation has been observed after myocardial infarction in the heart of mice [4] and it is also related to the initiation and progression of atherosclerosis [5]. TLR4 expression is augmented in cardiomyocytes from SHR (spontaneously hypertensive rats), when compared with Wistar-Kyoto rats, suggesting that this receptor may be implicated in hypertension-associated end-organ damage [6].

The downstream products of TLR signalling are COX (cyclo-oxygenase)-2 and pro-inflammatory cytokines [7]. COX-2 is an inducible isoform of the COX enzyme that catalyses the formation of PGs (prostaglandins), which may mediate inflammatory responses. One important PG produced by COX-1 and COX-2 is TXA₂ (thromboxane A₂), which is a potent vasoconstrictor that contributes to increased vascular contraction in arterial hypertension [8].

Arterial hypertension is a well-known risk factor for various CVDs and, currently, it is estimated that 25% of the adult population is affected by this disease [9]. It has been suggested that both innate and adaptive immunity contribute to the pathophysiology of hypertension [10]. In this regard, Guzik et al. [11] observed that mice lacking lymphocytes are resistant to the development of hypertension. Adoptive transfer of T-cells restored hypertensive responses to AngII and DOCA (deoxycorticosterone)-salt challenge in these mice [11], showing the importance of adaptive immune response in hypertension.

Considering the concept that inflammation is closely linked to hypertension and that the role of innate immune system, represented by TLR4 activation, in hypertension is still unclear, we hypothesized that TLR4 is up-regulated in resistance arteries of SHR and its increased activation contributes to both increased vascular response to contractile stimuli and augmented BP (blood pressure) levels in SHR.

MATERIALS AND METHODS

Animals

Male Wistar rats (15 weeks old) and SHR (5 and 15 weeks old; Harlan Laboratories) were used in the present study. All procedures were performed in accordance with the Guiding Principles in the Care and Use of Animals, approved by the Medical College of Georgia Committee on the Use of Animals in Research and Education and in accordance with the Guide for the Care and Use

of Laboratory Animals published by the U.S. National Institutes of Health. The animals were housed under a 12 h light/12 h dark cycle and fed on a standard chow diet with water *ad libitum*.

Treatment with the anti-TLR4 (anti-TLR4 antibody)

TLR4 inhibition was performed by treating 15-week-old SHR and Wistar rats with a daily intraperitoneal injection of 1 μ g of anti-TLR4 (rat monoclonal IgG_{2a}, saline-diluted; sc-13591, Santa Cruz Biotechnology) for 15 days [12]. Control SHR and Wistar rats were treated in the same way with 1 μ g of a non-specific IgG antibody (IgG_{2a}, saline-diluted; sc-2026, Santa Cruz Biotechnology) to rule out non-specific effects of TLR4 antibody treatment. In the text, the control groups are identified as IgG or non-specific IgG.

Arterial BP measurement

Rats were anaesthetized with a mixture of ketamine and xylazine (64.9 and 3.2 mg/kg of body weight respectively, intraperitoneal injections). The right carotid artery was cannulated with a heparinized polyethylene catheter (PE-50) that was exteriorized in the mid-scapular region. After 24 h, arterial pressure and HR (heart rate) were measured in conscious animals by a pressure transducer (model DT-100; Utah Medical Products) and recorded using an interface and software for computer data acquisition (Quad Bridge Amp/PowerLab 4/30; ADInstruments). HR was determined from the interbeat intervals.

Western blotting

Extracted proteins (50 μ g) from small mesenteric arteries were separated by electrophoresis on a 10% polyacrylamide gel and transferred on to a nitrocellulose membrane. Non-specific binding sites were blocked with 5% (w/v) non-fat dried skimmed milk powder in Tris-buffered saline (10 mmol/l Tris/HCl, pH 7.4, 100 mmol/l NaCl and 0.1% Tween 20) for 1 h at room temperature. Membranes were then incubated with primary antibodies overnight at 4°C. Primary antibodies were: anti-TLR4 (1:250 dilution), anti-COX-1 (1:500 dilution) and anti-COX-2 (1:500 dilution) (all antibodies from Cell Signaling Technology). Membranes were washed with Tris-buffered saline and incubated for 1 h at room temperature with the secondary anti-rabbit antibody (1:1500 dilution). After incubation, membranes were washed with Tris-buffered saline and the signals were revealed by chemiluminescence, visualized by autoradiography film and quantified by densitometry. The same membrane was used to determine β -actin protein expression using a monoclonal antibody against β -actin (1:15 000 dilution; Sigma-Aldrich), and its content was used to normalize protein expression in each sample. All representative Western blot images shown in Figures 1 and 5 were obtained from the same membrane.

Vascular function studies

After killing with isoflurane (via nasal 5% in 100% O₂), second-order mesenteric resistance arteries (200–300 μ m internal diameter) were removed and cleaned from fat tissue in Krebs solution (130 mmol/l NaCl, 14.9 mmol/l NaHCO₃, 4.7 mmol/l KCl, 1.18 mmol/l KH₂PO₄, 1.17 mmol/l MgSO₄·7H₂O, 1.56 mmol/l CaCl₂·2H₂O, 0.026 mmol/l EDTA and 5.5 mmol/l glucose). Arterial segments (2 mm in length) were mounted on 40 μ m wires in a small vessel myograph for isometric tension recording and equilibrated in Krebs solution for approximately 30 min, gassed with 5% CO₂ in O₂ to maintain a pH of 7.4. The relationship between resting wall tension and internal circumference was determined, and the internal circumference, L100, corresponding to a transmural pressure of 100 mmHg for a relaxed vessel *in situ*, was calculated. The vessels were set to the internal circumference L1, given by $L1 = 0.9 \times L100$. After stabilization, arterial integrity was assessed by stimulation of vessels, two times with 120 mmol/l KCl. Endothelial integrity was assessed by testing the relaxant effect of acetylcholine (1 μ mol/l; Sigma–Aldrich) on vessels pre-contracted with 3 μ mol/l NA (noradrenaline; Sigma–Aldrich).

Cumulative concentration–response curves to NA (10–100 μ mol/l) were performed in arteries with endothelium. Curves were performed in the presence and absence of either a COX-1 inhibitor {SC-560 [5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethylpyrazole]; 9 nmol/l} or COX-2 inhibitor {NS-398 [N-(2-cyclohexyloxy-4-nitrophenyl) methanesulfonamide]; 10 μ mol/l}, which were added to the preparation 30 min before starting the concentration–response curves to NA. Both inhibitors used are from Cayman Chemical.

Release of TXB₂ (thromboxane B₂) and 6-keto PGF_{1 α}

Mesenteric arteries were cut into transverse rings 4 mm in length, to measure the release of prostanoids. These were placed for 30 min in siliconized tubes containing 0.5 ml of Krebs solution at 37°C, and stimulated with 100 mM NA for 15 min. The PGs were measured with a commercially available EIA kit (Cayman Chemical). Twice-diluted 50 μ l samples were used for measurement of TXB₂ (a stable metabolite of TXA₂) and 6-keto PGF_{1 α} (stable metabolite of PGI₂). The assays were performed according to the manufacturer's instructions. The amounts of PGs released are expressed as pg or ng/mg of wet mass of mesenteric artery.

Cytokine measurement

Serum levels of IL-6 (interleukin-6) and TNF α (tumour necrosis factor α) were determined using a quantitative

sandwich EIA/ELISA kits (GE Healthcare) in IgG- and anti-TLR4-treated SHR. IL-6 and TNF α concentrations are expressed as pg/ml.

Data analysis

Results are shown as means \pm S.E.M. and *n* represents the number of animals used in the experiments. Contractile responses are expressed as the maximum response produced by each agonist concentration. Concentration–response curves were fitted using a non-linear interactive fitting program (Graph Pad Prism 4.0) and two pharmacological parameters were analysed: the maximal effect elicited by the agonist (E_{\max}) and the sensitivity to this agonist ($-\log EC_{50}$, pD_2). Statistical analyses of E_{\max} and pD_2 values were performed using one-way ANOVA (post-hoc Tukey) or Student's *t* test, where appropriate. Values of $P < 0.05$ were considered statistically significant.

RESULTS

TLR4 expression in mesenteric resistance arteries from SHR and Wistar rats

TLR4 protein expression was significantly increased in mesenteric arteries from 15-week-old SHR (hypertensive phase) compared with same age Wistar or 5-week-old SHR (pre-hypertensive phase) (Figure 1A and 1B respectively). Treatment of 15-week-old SHR and Wistar with anti-TLR4 antibody decreased TLR4 expression in mesenteric resistance arteries from SHR, but not in Wistar rats (Figure 1C). These results demonstrate that TLR4 expression is increased in SHR and that the anti-TLR4 treatment was effective in reducing its expression.

Effect of anti-TLR4 treatment on haemodynamic parameters and body mass

Anti-TLR4 treatment lowered MAP (mean arterial pressure) in SHR, compared with SHR treated with non-specific IgG (Figure 2A). However, no change in MAP was observed in Wistar rats after anti-TLR4 treatment. HR (Figure 2B) was similar between all the groups. Body mass did not change among the groups (510 \pm 15 and 502 \pm 30 g in the IgG and anti-TLR4 Wistar groups respectively; 334 \pm 8 and 327 \pm 13 g in the IgG and anti-TLR4 SHR groups respectively). These results suggest that increased activation of TLR4 contributes to augmented BP observed in SHR.

Effect of anti-TLR4 treatment on vascular contractility

The E_{\max} and pD_2 to NA were significantly decreased in endothelium-intact mesenteric resistance arteries from anti-TLR4-treated SHR when compared with those in

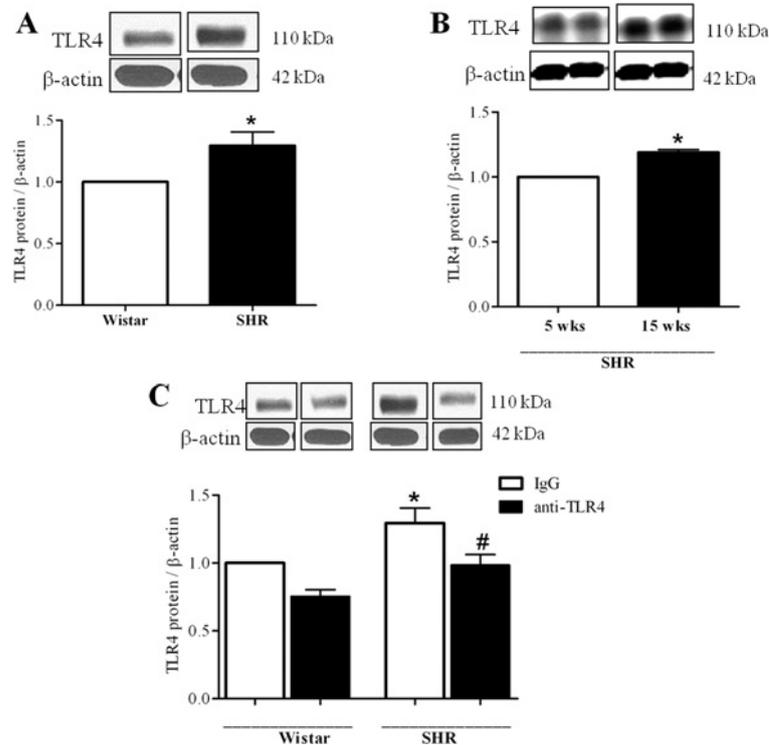


Figure 1 TLR4 protein expression is augmented in mesenteric resistance arteries from 15-week-old SHR compared with age-matched Wistar rats or 5-week-old SHR

Anti-TLR4 treatment decreased TLR4 protein expression in SHR. (A) TLR4 protein expression was analysed in mesenteric resistance arteries from 15-week-old Wistar rats (white bar) and SHR (black bar), and (B) in 5-week-old (white bar) and 15-week-old (black bar) SHR. (C) We also evaluated TLR4 protein expression in Wistar rats and SHR treated with IgG or anti-TLR4. Above the histograms are representative Western blot images of TLR4 and β -actin protein expression. Histograms show the relative expression of TLR4 after normalization to β -actin expression. Values are means \pm S.E.M., $n = 6$. * $P < 0.05$ compared with Wistar rats (A), 5-week-old SHR (B) and Wistar rats IgG (C); # $P < 0.05$ compared with SHR IgG (C). Statistical tests, Student's t test (A and B) and one-way ANOVA (C).

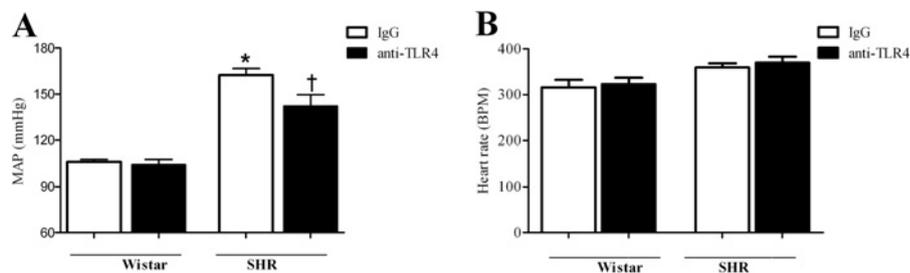


Figure 2 Anti-TLR4 treatment decreased the BP of SHR

(A) MAP (mmHg) and (B) HR [beats/min (BPM)] were evaluated in Wistar rats and SHR treated with IgG (white bars) or anti-TLR4 (black bars). Results are expressed as means \pm S.E.M., $n = 6$. † $P < 0.05$ compared with Wistar IgG and ‡ $P < 0.05$ compared with SHR IgG, using a one-way ANOVA.

arteries from unspecific IgG-treated SHR (Figures 3A and 3C and Table 1). No differences were observed between arteries from the Wistar groups (Figure 3B).

Pre-incubation of mesenteric resistance arteries with either SC-560 (COX-1 inhibitor) or NS-398 (COX-2 inhibitor) decreased the E_{max} (maximal response) and pD_2

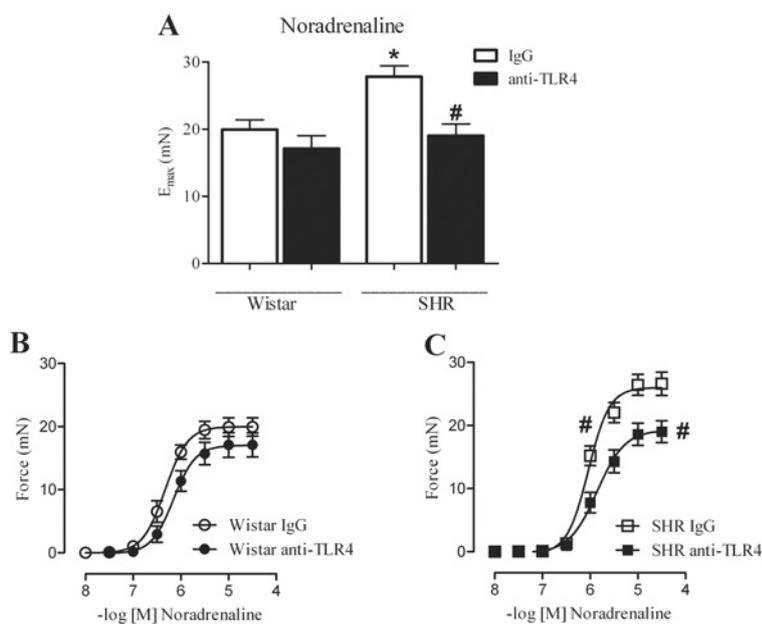
($-\log EC_{50}$) to NA in arteries from IgG-treated SHR, but not in arteries from anti-TLR4-treated SHR and Wistar groups (Figures 4A and 4B and Table 1).

Together, these findings demonstrate that anti-TLR4 treatment decreases the augmented contractile response observed in SHR. In addition, COX-1 and COX-2

Table 1 E_{\max} (maximal response) and sensitivity (pD_2) to KCl and NA with or without inhibitors in mesenteric resistance arteries from Wistar and SHR treated with IgG or anti-TLR4

Values are means \pm S.E.M., $n = 6-12$. * $P < 0.05$ compared with SHR + IgG (NA); # $P < 0.05$ compared with Wistar + IgG (NA) using a one-way ANOVA. NS-398, COX-2 inhibitor; SC-560, COX-1 inhibitor.

Treatment	Wistar + IgG		Wistar + anti-TLR4		SHR + IgG		SHR + anti-TLR4	
	E_{\max}	pD_2	E_{\max}	pD_2	E_{\max}	pD_2	E_{\max}	pD_2
NA	19.9 \pm 1.4	6.3 \pm 0.06	17.1 \pm 1.9	6.1 \pm 0.08	27.8 \pm 1.6#	6.1 \pm 0.04	19.0 \pm 1.7*	5.8 \pm 0.08*
NA + NS-398	14.2 \pm 1.9	6.0 \pm 0.07	14.9 \pm 1.7	5.9 \pm 0.06	17.1 \pm 1.3*	5.2 \pm 0.05*	15.8 \pm 1.1	5.5 \pm 0.06
NA + SC-560	21.3 \pm 3.7	6.1 \pm 0.08	19.5 \pm 1.9	6.3 \pm 0.07	15.9 \pm 1.1*	5.3 \pm 0.04*	18.9 \pm 1.5	5.5 \pm 0.03

**Figure 3** NA-induced vasoconstriction in mesenteric resistance arteries from SHR is attenuated by anti-TLR4 treatment

Maximal response to (A) NA in mesenteric resistance arteries from Wistar rats and SHR treated with IgG (white bars) or anti-TLR4 (black bars). Cumulative concentration-response curves to NA in endothelium-intact mesenteric resistance arteries from (B) Wistar treated with IgG (open circle) or anti-TLR4 (closed circle) and (C) SHR treated with IgG (open square) or anti-TLR4 (closed square). Each point represents the means \pm S.E.M. of maximal response to each concentration, $n = 10-12$. * $P < 0.05$ compared with Wistar IgG, and # $P < 0.05$ compared with SHR IgG, using a one-way ANOVA.

are involved in signalling pathways that contribute to decreased NA contraction response in mesenteric resistance arteries from anti-TLR4-treated SHR.

Effect of anti-TLR4 treatment on COX expression, pro-inflammatory cytokines and prostanoids release

We evaluated whether TLR4 modulates, in SHR, the expression of COX-1 and COX-2, as well as the release of the prostanoids TXA₂ and prostacyclin by mesenteric arteries and the levels of the cytokines IL-6 and TNF α , in the serum.

COX-1 protein expression in small mesenteric arteries did not change after anti-TLR4 treatment when compared

with IgG groups (Figure 5A). However, COX-2 protein expression was decreased in mesenteric arteries from SHR treated with anti-TLR4 compared with IgG-treated SHR (Figure 5B).

The TXB₂ released by segments of mesenteric arteries stimulated with NA (100 mM) is increased in SHR IgG when compared with all other groups, and the anti-TLR4 treatment decreased the production of this vasoconstrictor prostanoids in SHR (Figure 5C). No difference was found in 6-keto PGF_{1 α} secretion between the groups (Figure 5D).

Serum levels of IL-6 and TNF α were measured in IgG-treated and anti-TLR4-treated SHR. IL-6, but not TNF α , was decreased after treatment with anti-TLR4 in SHR (IL-6, 478 \pm 44.5 and 337 \pm 24 pg/ml in the IgG and

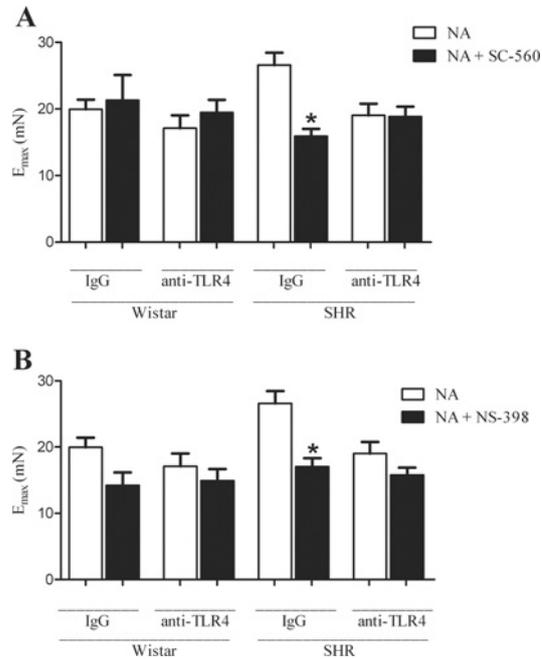


Figure 4 COX-1 and COX-2 inhibition decreased the maximal response to NA only in IgG-treated SHR mesenteric arteries

E_{max} to NA in the absence (white bars) or presence of (A) SC-560 (COX-1 inhibitor) or (B) NS-398 (COX-2 inhibitor) (black bars) in mesenteric resistance arteries from Wistar rats and SHR treated with IgG or anti-TLR4. * $P < 0.05$ compared with SHR IgG NA, using a one-way ANOVA.

anti-TLR4 groups respectively, $P < 0.05$, using a Student's t test; TNF α , 112 ± 53 and 98 ± 9.5 pg/ml in the IgG and anti-TLR4 groups respectively; values are means \pm S.E.M., $n = 9$).

These results demonstrate that TLR4 inhibition attenuated the activation of inflammatory markers, as demonstrated by decreased COX-2 expression, lower IL-6 serum levels and thromboxane release in mesenteric arteries from SHR.

DISCUSSION

The major findings of the present study are that the treatment of adult SHR with anti-TLR4 antibody reduces MAP as well as decreasing contractile responses in mesenteric resistance arteries when compared with non-specific IgG-treated SHR.

Essential hypertension is characterized by increased peripheral vascular resistance, which is mainly determined by small resistance arteries and arterioles. At the functional level, both an increase in the contractile responses or a decrease in relaxant responses in resistance arteries might increase peripheral resistance. Inflammation of the vascular wall is associated with

the dysfunction of blood vessels [13]. In view of the decreased BP observed after anti-TLR4 treatment in SHR, we investigated whether this treatment could improve contractile responses in resistance arteries from SHR. Our results demonstrate that mesenteric resistance arteries from anti-TLR4-treated SHR showed lower sensitivity to NA compared with IgG-treated SHR. However, no changes were found in Wistar groups. These findings suggest that the innate immune response, represented by TLR4, plays a role in the vascular dysfunction associated with hypertension [13].

Activation of TLR4 results in an increase in its own expression [14]. Therefore the reduction of TLR expression is a possible mechanism by which TLR activation can be controlled [15,16]. Our results demonstrated that anti-TLR4 treatment decreased TLR4 protein expression, which is up-regulated in adult SHR compared with young SHR and compared with Wistar rats. The regulation of TLR4 expression was showed in a model of myocardial ischaemia/reperfusion, in which TLR4 is up-regulated. In those rats, treatment with valsartan, an angiotensin receptor blocker with anti-inflammatory properties, decreased the expression and activation of TLR4 [17].

Anti-TLR4 antibody treatment has been tested in some inflammatory conditions, such as sepsis and chronic bowel disease, with promising results, showing a decrease in the inflammatory process [18,19]. Hypertension is considered as a chronic inflammatory disease, in which elevated pro-inflammatory cytokines and COX-derived prostanoids, mainly from the COX-2 isoform, are observed [20]. We hypothesized that the reduction in TLR4 effects, by using neutralizing antibody *in vivo*, would ameliorate hypertensive low-grade inflammation. Here, we observed that treatment with anti-TLR4 antibody decreased the expression of TLR4, COX-2 and IL-6 serum levels, which are markers of inflammation. Therefore our results suggest that TLR4 is associated with low-grade inflammation in hypertension.

The major downstream molecular mechanism involved in TLR4 activation is a MyD88 (myeloid differentiation factor 88)-dependent pathway that involves IRAK (IL-1-receptor-associated kinase), TRAF-6 (TNF-receptor-associated factor 6) and MAPK (mitogen-activated protein kinase), and that culminates in the activation of NF- κ B [2]. In turn, NF- κ B mediates the transcription of COX-2 and pro-inflammatory cytokine genes. Kuper et al. [21] demonstrated that LPS mediates enhanced COX-2 expression in renal medullary collection duct cells by TLR4-mediated activation of the NF- κ B signalling pathway. In hypertension, as mentioned before, TLR4 expression is increased in the heart of SHR compared with Wistar-Kyoto rats [6]. Conversely, studies such as the one by Li et al. [22] showed that NF- κ B is activated in the heart, kidney and aorta in SHR compared with Wistar-Kyoto rats.

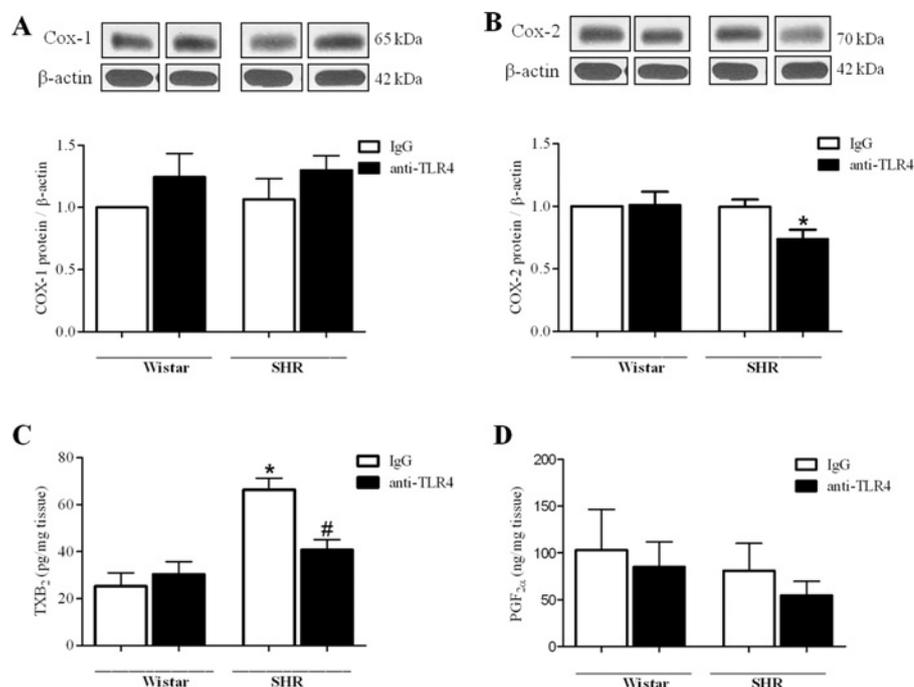


Figure 5 Anti-TLR4 treatment decreases COX-2 protein expression and TXB₂ release in mesenteric arteries

(A) COX-1 and (B) COX-2 proteins expression in mesenteric resistance arteries from IgG- (white bars) or anti-TLR4-treated (black bars) Wistar rats and SHR. Upper panel, representative Western blot images of COX-1 (A) and COX-2 (B) protein expression. The histograms show the relative expression of COX-1 and COX-2 after normalization to β -actin expression. Release of (C) TXB₂ and (D) 6-keto PGF_{1 α} by mesenteric arteries stimulated with 100 μ M NA. Each bar represents the means \pm S.E.M., $n = 5-6$. * $P < 0.05$, using a one-way ANOVA.

Many studies have shown that TLR4 is clearly responsible for inflammation induced by endogenous ligands, such as CRP (C-reactive protein) [23] and HSP60 and HSP70 in smooth muscle cells and many other cell types [24,25]. In hypertension, these molecules are increased [26,27] and could act as long-term TLR4 activators, resulting in augmented expression of several pro-inflammatory cytokines in vascular smooth muscle [28]. Pro-inflammatory cytokines regulate the expression and function of several proteins, including adhesion molecules, MAPKs, extracellular matrix components and growth factors, which are important in vessel hypertrophy and vascular dysfunction described in hypertension [13,29]. Cytokines seem to influence the balance between vasoconstrictor and vasodilator factors, as well as regional differences in the release and responsiveness to these factors, contributing to the increased responsiveness within a specific vascular bed in hypertension [20].

IL-6 is a pro-inflammatory cytokine that is released from numerous cell types, including endothelial cells [30,31], VSMCs (vascular smooth muscle cells) [32] and macrophages [33]. IL-6 stimulates the synthesis of many acute-phase reaction proteins, including CRP, SAA (serum amyloid A) and fibrinogen [34]. IL-6 also promotes VSMC proliferation, a hallmark of

hypertension and atherosclerosis [35]. Some studies have shown a positive association between IL-6 levels and high BP [36-38]. Previous work from Brands and co-workers [39] showed that hypertension caused by a high-salt diet and AngII was blunted in mice lacking a functional gene for IL-6. In our present study, we observed a reduction in IL-6 secretion in SHR by anti-TLR4 treatment, which was accompanied by a decrease in BP, suggesting that IL-6 is a key cytokine in the development of the pathophysiology of hypertension.

Evidence showed that the vasoconstrictor response to adrenergic agonists is largely mediated by COX-derived vasoconstrictor prostanoids [8,40]. Hypertension is thought to modify the role of these products in the vasodilator and vasoconstrictor responses [8,41,42]. Furthermore, it has been reported that activation of TLR4 by LPS and AngII may induce the release of prostanoids in cultured macrophages and smooth muscle cells [28,43]. In the present study, we demonstrated that COX-2 protein expression is decreased in mesenteric resistance arteries from anti-TLR4-treated SHR compared with IgG-treated SHR, and no differences were found in COX-1 expression among groups. Despite the lack of change in the expression of COX-1, one possibility is that its activity may be decreased after anti-TLR4 therapy. This hypothesis is supported by the fact that the

inhibition of COX-1 and COX-2 reduced the contractile response to NA only in mesenteric resistance arteries from IgG-treated SHR, whereas it remained unchanged in resistance arteries from anti-TLR4-treated SHR. In addition, the release of TXA₂ in mesenteric arteries after NA stimulation was reduced in anti-TLR4-treated SHR compared with SHR treated with IgG. Taken together, these results suggest that the innate immune response, represented by TLR4 activation, plays a role in the vascular dysfunction associated with hypertension by a COX-dependent mechanism [13]. Future studies will help clarify the molecular pathways linking these events.

Hypertension is an important worldwide public-health challenge because of its high prevalence and concomitant risks of cardiovascular and kidney disease. A variety of pharmacological preparations are available for therapy; however, despite these options, vascular dysfunction persists in many patients, and end-organ injury remains a serious complication. The cardiovascular system is exposed to pathogens and danger signals (endogenous ligands), resulting in activation of pattern-recognition receptors including TLRs. Considering that hypertension is a low-grade inflammatory disease, the innate immune response, represented mainly by TLR, may be investigated as a mechanism that contributes to the development of this condition.

In conclusion, our present findings demonstrate that increased TLR4 expression may play an important role in arterial hypertension. Moreover, anti-TLR4 treatment decreased BP, pro-inflammatory mediators and vascular contraction in resistance arteries of SHR.

AUTHOR CONTRIBUTION

Experimental work was performed by Gisele Bomfim, Rosângela Dos Santos, Maria Aparecida Oliveira and Fernanda Giachini. Design, analysis and interpretation of the data were performed by Eliana Akamine, Rita Tostes and Zuleica Fortes Clinton Webb and Maria Helena Carvalho, who directed the study.

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