Acute Toll-like Receptor 4 Activation Impairs Rat Renal Microvascular Autoregulatory Behaviour

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Abstract

Aim—Little is known about how toll-like receptor 4 (TLR4) influences the renal microvasculature. We hypothesized that acute TLR4 stimulation with lipopolysaccharide (LPS) impairs afferent arteriole autoregulatory behaviour, partially through reactive oxygen species (ROS).

Methods—We assessed afferent arteriole autoregulatory behaviour after LPS treatment (1 mg kg^{-1}; i.p.) using the *in vitro* blood perfused juxtamedullary nephron preparation. Autoregulatory behaviour was assessed by measuring diameter responses to step-wise changes in renal perfusion pressure. TLR4 expression was assessed by immunofluorescence, immunohistochemistry and western blot analysis in the renal cortex and vasculature.

Results—Baseline arteriole diameter at 100 mmHg averaged 15.2 ± 1.2 μm and 12.2 ± 1.0 μm for control and LPS groups (P<0.05), respectively. When perfusion pressure was increased in 15 mmHg increments from 65 to 170 mmHg, arteriole diameter in control kidneys decreased significantly to $69 \pm 6\%$ of baseline diameter. In the LPS treated group, arteriole diameter remained essentially unchanged ($103 \pm 9\%$ of baseline), indicating impaired autoregulatory behaviour. Pre-treatment with anti-TLR4 antibody or the TLR4 antagonist, LPS-RS, preserved autoregulatory behaviour during LPS treatment. P2 receptor reactivity was normal in control and LPS treated rats. Pre-treatment with Losartan (angiotensin type 1 receptor blocker; (AT_1) 2 mg kg^{-1}; i.p.), increased baseline afferent arteriole diameter but did not preserve autoregulatory behaviour in LPS treated rats. Acute exposure to Tempol (10^{-3} mol L^{-1}), a superoxide dismutase mimetic, restored pressure-mediated vasoconstriction in kidneys from LPS-treated rats.

Conclusion—These data demonstrate that TLR4 activation impairs afferent arteriole autoregulatory behaviour, partially through ROS, but independently of P2 and AT_1 receptor activation.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest in relation to the publication of the present study.

INTRODUCTION

Renal autoregulation is essential for maintaining stable renal blood flow (RBF) and glomerular capillary pressure over a wide range of arterial pressure. Autoregulation of renal hemodynamics is accomplished by precise and automatic pressure-mediated resistance adjustments of the preglomerular vasculature [1, 2]. Loss of autoregulatory efficiency can lead to inappropriate transmission of arterial pressure downstream to the glomerular capillaries. Sustained increases in glomerular capillary pressure lead to renal pathologies such as glomerular sclerosis, chronic kidney disease (CKD) and renal failure [2–4].

Acute kidney injury (AKI) during endotoxemia is characterized by increased renal vascular resistance and reduced RBF [5]. In septic patients, the increased plasma Ang II levels reportedly correlate with microvascular dysfunction [6]. In rodent models, endotoxemia reduces RBF and glomerular filtration rate (GFR), while increasing preglomerular vascular resistance [5, 7–10]. Boffa et al. demonstrated that lipopolysaccharide (LPS) treatment decreased mean arterial pressure, RBF, and GFR, while increasing renal vascular resistance. Notably, renal vascular sensitivity to angiotensin II (Ang II), norepinephrine (NE), and L-N-Nitroarginine methyl ester (L-NAME), was normal compared to control mice [5]. Endotoxemic rats exhibited increased afferent arteriole resistance with no change in efferent arteriole resistance [8]. Furthermore, acute administration of the angiotensin type 1 (AT$_1$) receptor blocker candesartan, after LPS treatment, significantly increased RBF [11]. These data suggest that the sensitivity of the renal vasculature to vasoactive agonists is relatively normal in endotoxemia such that the elevated plasma Ang II levels in endotoxemia leads to renal vasoconstriction and reduction of RBF and GFR.

Toll-like receptors (TLRs) belong to a family of innate immune receptors that recognize pathogen-associated molecular patterns (PAMPs), and/or damage-associated molecular patterns (DAMPs) [12, 13]. There are currently eleven human, and thirteen murine TLRs [13]. The TLR family is separated into two distinct categories; 1) those that localize to the cell membrane and 2) those that localize to intracellular endosomes. PAMPs and DAMPs can activate TLRs, which in turn stimulate the innate immune system [12, 13]. TLRs can signal through the MyD88-dependent pathway, using MyD88 as an adapter protein, and stimulate production of pro-inflammatory cytokines [14]. Toll-like receptor 4 (TLR4), can also utilize a MyD88-indpendent signalling pathway using TRIF as an adapter protein [14]. TLRs are expressed on immune cells, (monocytes/macrophages, B and T lymphocytes, dendritic cells), smooth muscle and endothelial cells [15, 16]. Within the kidney, glomeruli and tubular epithelial cells express TLR1, TLR2, TLR3, TLR4 and TLR6 [17, 18]. LPS, a component of gram negative bacteria, is a well-accepted ligand for TLR4 [19]. Studies indicate that TLR4 mediates endotoxin-induced acute renal failure through rapid release of tumour necrosis factor (TNF), leading to renal neutrophil infiltration and apoptosis [20]. In MyD88$^{-/-}$ mice, TLR4 is unresponsive to LPS as measured by improved survival rate and reduced pro-

inflammatory mediators [14, 21, 22]. Dear et al. employed a more severe sepsis model and found that acute renal failure involved MyD88-dependent signalling [23]. Furthermore, recent studies revealed that TLR4 can also be activated by non-infectious DAMPs, such as high mobility group box-1, fibrinogen, and heat shock proteins in acute and chronic inflammatory pathologies [12, 24–27]. Finally, TLR4 directly correlates with inflammatory markers and renal disease severity, suggesting a pathogenic role for TLR4 in CKD patients [28].

Stimulation of TLR4 impairs vascular function in peripheral vascular beds [26, 29–31]. In hypertensive models, anti-TLR4 antibody treatment prevents vascular dysfunction in aorta and mesenteric arteries by supressing TNF-α, IL-6, and MCP-1 and reactive oxygen species (ROS) production [26, 27]. Other studies in rodent models showed that impaired vascular function involves TLR4-dependent NADPH-oxidase generation of ROS [27, 30]. Selective TLR4 blockade supressed MyD88 expression, NF-κB translocation, and ROS production in mesenteric arteries from STZ-induced diabetic rats [32]. Thus, TLR4 activation can induce vascular dysfunction by increased oxidative stress in inflammatory pathologies such as atherosclerosis, diabetes, hypertension, and metabolic syndrome.

There is growing evidence that TLR4 stimulation facilitates progression of AKI and CKD [33, 34]. Immune system activation and inflammation exert important influences on renal microvascular function. Both acute and chronic renal inflammation impair afferent arteriole autoregulatory behaviour in pathological conditions [35–37]. Renal autoregulatory behaviour was preserved in Ang II hypertensive rats treated with the anti-inflammatories, pentosan polysulfate or mycophenolate mofetil, despite persistent hypertension [36, 38]. Sharma et al. showed that acute exposure to TGF-β impairs afferent arteriole autoregulatory behaviour, and this impairment was reversed by acute Tempol treatment. These data suggest that inflammatory processes and oxidative stress, contribute to impaired renal autoregulatory efficiency in pathological conditions.

This study was designed to test the hypothesis that acute TLR4 stimulation impairs afferent arteriole autoregulatory behaviour in part by stimulating oxidative stress. To assess this, we acutely stimulated TLR4 with a low dose of a TLR4 selective LPS and determined its effect on afferent arteriole autoregulatory behaviour and reactivity. We also determined whether, or not, inhibition of TLR4 actions with either anti-TLR4 antibody, or a competitive antagonist of TLR4, LPS-RS, preserved autoregulatory behaviour. For this purpose, we determined the functionality of voltage-gated calcium channels, α-adrenergic receptors, P2 receptors, and AT_1 receptors in untreated and acute LPS treated rats. P2 receptors, specifically $P2X_1$ are involved in efficient afferent arteriole autoregulatory behaviour [39]. The physiological role of TLR4 activation on afferent arteriole reactivity and overall autoregulatory behaviour has not been investigated. The results indicate that acute TLR4 activation impairs afferent arteriole autoregulatory behaviour through mechanisms that involve Tempol-sensitive mediators of oxidative stress, but are independent of P2 and AT_1 receptor activation.

RESULTS

Acute LPS treatment significantly increases renal TLR4 expression

Renal TLR4 expression was assessed using DAB staining in whole kidney slices from control and LPS treated rats. In control kidneys, TLR4 was expressed in the cortex, with strong expression in the renal tubules (Fig. 1a, left panels). In the LPS group, TLR4 expression increased based on higher intensity brown staining (TLR4) compared to the control group (Fig. 1a, right panels). TLR4 expression increased significantly (Fig. 1b) in the LPS group (7.7 ± 0.3% of total kidney area; P<0.05 vs. control) compared to control kidneys (2.5 ± 0.3% of total kidney area).

TLR4 is expressed in renal vascular smooth muscle cells

To determine whether TLR4 protein is expressed in renal vascular smooth muscle cells, we looked for co-localization of immunofluorescence for TLR4 and α-actin in OCT frozen kidneys from control and acute 4-hour LPS treated rats using confocal immunofluorescence microscopy. TLR4 was strongly expressed in the renal tubules, and Figure 1c illustrates that TLR4 was also detected in the renal microvasculature by expression of TLR4 (red) in the same cells that expressed α-smooth muscle actin (green), in both the control (top panels) and LPS groups (bottom panels). These images reveal that TLR4 is expressed in renal vascular smooth muscle cells.

The effect of acute LPS treatment on SBP

Conscious baseline SBPs (n=12/group) were similar between control (136 ± 2 mmHg) and LPS (131 ± 1 mmHg) groups (Table 1). LPS treatment significantly decreased SBP within 60 minutes (113 ± 2 mmHg) and persisted throughout the 4-hour observation period.

Acute TLR4 activation with LPS impaired afferent arteriole autoregulatory behaviour

Experiments were performed to assess the impact of TLR4 activation on afferent arteriole autoregulatory behaviour during acute LPS treatment (Fig. 2). Baseline diameters at 100 mmHg averaged 15.2 ± 1.2 and 12.2 ± 1.0 μm for control and LPS groups, respectively (P<0.05 vs. control; Fig. 2a). Reducing perfusion pressure from 100 to 65 mmHg increased afferent arteriole diameter to 112 ± 2% of baseline (P<0.05) in the control group (Fig. 2b), but to just 103 ± 9% of baseline in the LPS group (P<0.05 vs. control). When perfusion pressure was increased from 65 to 170 mmHg, in 15 mmHg increments, the control group exhibited stepwise reductions in diameter ultimately reaching 69 ± 6% of baseline at 170 mmHg (P<0.05). In contrast, arteriole diameter in the LPS treated group remained essentially unchanged at 170 mmHg, demonstrating impaired pressure-induced vasoconstriction. These data indicate that LPS treatment impairs afferent arteriole autoregulatory behaviour.

TLR4 blockade with LPS-RS preserved autoregulatory behaviour

Experiments were performed to assess the impact of TLR4 blockade on autoregulatory behaviour during acute LPS treatment. Rats were co-treated with LPS and a competitive TLR4 antagonist, LPS-RS, 4 hours prior to kidney harvesting. As shown in Figure 2a,

baseline afferent arteriole diameter in the LPS + LPS-RS group at 100 mmHg averaged 14.5 ± 0.6 μm (P<0.05 LPS vs. LPS + LPS-RS). When perfusion pressure was decreased to 65 mmHg, arteriole diameter in the LPS + LPS-RS group increased to 106 ± 2% of baseline diameter (P<0.05, Fig. 2b). Increasing perfusion pressure from 65 to 170 mmHg, decreased arteriole diameter in the LPS + LPS-RS group to 86 ± 4% of the baseline diameter (P<0.05, LPS vs. LPS + LPS-RS), similar to the control group. These data demonstrate that TLR4 blockade preserves afferent arteriole autoregulatory behaviour during LPS treatment.

Pre-treatment with anti-TLR4 antibody preserved afferent arteriole autoregulatory behaviour during acute LPS treatment

Experiments were performed to assess the impact of TLR4 inhibition with anti-TLR4 antibody treatment on afferent arteriole autoregulatory behaviour during acute LPS treatment (Fig. 3). Rats received anti-TLR4 antibody treatment 12 hours prior to the bolus injection of LPS. Kidneys were harvested 4 hours later for evaluation of afferent arteriole autoregulatory behaviour. As shown in Figure 3a, baseline diameters averaged 15.6 ± 0.8 μm in the LPS + anti-TLR4 group. When perfusion pressure was reduced from 100 to 65 mmHg, afferent arteriole diameter in the LPS + anti-TLR4 group increased to 106 ± 3% of baseline diameter (Fig. 3b). Increasing perfusion pressure from 65 to 170 mmHg, the LPS + anti-TLR4 group demonstrated pressure-mediated vasoconstriction, virtually indistinguishable from control kidneys.

To control for nonspecific antibody effects, we treated a separate group with LPS + IgG$_{2a}$. Baseline diameter at 100 mmHg averaged 13.0 ± 0.4 μm (Fig. 3a) in the LPS + IgG$_{2a}$ group, which was similar to the LPS alone group but significantly smaller than the control or anti-TLR4 groups. When perfusion pressure was decreased to 65 mmHg, afferent arteriole diameter remained unchanged (Fig. 3b). Increasing perfusion pressure from 65 to 170 mmHg, produced no detectable change in diameter in the LPS + IgG$_{2a}$ group. These data demonstrate that anti-TLR4 antibody treatment preserves afferent arteriole autoregulatory behaviour despite a LPS challenge. Similar protection was not achieved with IgG$_{2a}$ indicating that the protective effect is TLR4 specific.

Afferent arteriole responses to KCl and NE were normal in acute LPS treated rats

Autoregulatory resistance adjustments require functional voltage-dependent calcium channels [40, 41]. Since LPS treatment impaired autoregulatory behaviour, we assessed afferent arteriole responses to depolarization with KCl (55 mmol L^{-1}), and α-adrenergic stimulation with NE (100 nmol L^{-1}). Baseline arteriole diameters at 100 mmHg were similar between groups and averaged 15.9 ± 1.1 μm for control and 14.9 ± 1.2 μm in the LPS group. The response to KCl was nearly identical in both groups. Arteriole diameter decreased to 56 ± 4% of baseline diameter (P<0.05) in the control group and 56 ± 3% of baseline (P<0.05) in the LPS group.

Baseline diameters during assessment of NE (100 nmol L^{-1}) averaged 11.9 ± 1.0 μm compared to the control group (16.3 ± 0.9 μm; P<0.05). Afferent arteriole responses to NE were essentially identical in the control (70 ± 2%) and LPS groups (72 ± 3%). Together,

these data indicate that voltage-gated L-type calcium channels, and α-adrenergic receptors are functioning normally in rats receiving LPS treatment.

Afferent arteriole P2, P2X$_1$, and P2Y$_2$ receptor responses were normal during acute LPS treatment

P2 receptors play an important role in afferent arteriole autoregulatory behaviour in normal conditions and in pathological settings, such as high salt diet and hypertension [42–45]. Therefore, we examined afferent arteriole reactivity to P2, P2X$_1$ and P2Y$_2$ receptor activation using ATP, β,γ-methylene ATP (β,γ-mATP), and UTP, respectively. Figure 4a illustrates afferent arteriole reactivity to ATP (10^{-8}–10^{-4} mol L^{-1}). Baseline diameters at 100 mmHg averaged 15.1 ± 0.9 μm and 12.4 ± 0.8 μm in the control and LPS groups, respectively (P<0.05 vs. control). Increasing concentrations of ATP reduced arteriole diameter to $68 \pm 5\%$ (P<0.05 vs. baseline) and $76 \pm 5\%$ of baseline diameter (P<0.05 vs. baseline) in the control and LPS groups, respectively (Fig. 4a). The magnitude of the ATP-mediated responses were similar between the control and LPS groups, demonstrating that general P2 receptor function is not significantly changed in afferent arterioles of LPS treated rats.

Inscho et al. reported that P2X$_1$ receptor blockade or genetic deletion of P2X$_1$ receptor (P2X$_1$$^{-/-}$ mice) impairs afferent arteriole autoregulatory behaviour, indicating that P2X$_1$ receptors are important for renal autoregulatory efficiency [39, 46, 47]. P2X$_1$ receptor function was assessed by determining arteriole responses to the selective P2X$_1$ agonist, β,γ-mATP (10^{-8}–10^{-4} mol L^{-1}). β,γ-mATP evoked nearly identical concentration-dependent vasoconstriction in the control and LPS groups (Fig. 4b). The similarity of the responses to β,γ-mATP between the two groups, suggests that renal microvascular P2X$_1$ receptor function is intact in LPS treated rats.

Afferent arterioles are also responsive to P2Y$_2$ receptor stimulation with UTP [43, 48], so we determined the effect of LPS treatment on afferent arteriole reactivity to UTP (10^{-8}–10^{-4} mol L^{-1}). Baseline diameter at 100 mmHg was significantly smaller in the LPS group (P<0.05 vs. control). UTP decreased afferent arteriole diameter similarly in both the control and LPS groups indicating P2Y$_2$ receptor function is not significantly altered by LPS treatment (Fig. 4c). Together with the KCl and NE findings, these data suggest that arterioles from the LPS treated group can vasoconstrict normally to some stimuli, but they do not respond appropriately to changes in perfusion pressure.

Effect of AT$_1$ receptor blockade on SBP and impairment of autoregulatory behaviour in LPS treated rats

In the Losartan treated groups, baseline SBP's were similar between control + Losartan (136 ± 1 mmHg) and LPS + Losartan (136 ± 1 mmHg) groups prior to Losartan and/or LPS treatment (Table 2). Losartan administration significantly decreased SBP in both groups in the first hour. In the control + Losartan group, SBP remained significantly lower over the 5-hour treatment period compared to baseline (Table 2). LPS treatment began after the first hour of Losartan treatment in the LPS + Losartan group. LPS treatment decreased SBP further (P<0.05 vs Losartan alone) in hour 2 (110 ± 1 mmHg; P<0.05), and this decrease

was maintained throughout the combined Losartan + LPS treatment period (Table 2). These data indicate that AT_1 receptor blockade decreases SBP in control and LPS treated rats and the effects of Losartan and LPS on SBP are additive.

Plasma Ang II levels are increased in septic patients and correlate with microvascular dysfunction [6]. Therefore, to control for the effects of Ang II on the renal microvasculature in our acute LPS treated model, we included two additional groups control + Losartan (2 mg kg^{-1}; i.p.) and LPS + Losartan and assessed autoregulatory behaviour. Losartan treatment increased baseline diameter in kidneys from control + Losartan (18.3 ± 1.2 μm) and LPS + Losartan treated rats (15.8 ± 0.6 μm; P<0.05 vs. LPS; Fig. 5a). Reducing renal perfusion pressure from 100 to 65 mmHg increased arteriole diameter to 109 ± 1% of baseline diameter in the control + Losartan group (P<0.05), but the LPS + Losartan group remained essentially unchanged (Fig. 5b). When renal perfusion pressure was increased from 65 to 170 mmHg, the control + Losartan group exhibited pressure-mediated reductions in afferent arteriole diameter reaching 75 ± 2% of baseline at 170 mmHg (P<0.05; Fig. 5b). In contrast, arteriole diameter in the LPS + Losartan group remained essentially unchanged, reaching 98 ± 2% of baseline diameter at 170 mmHg (Fig. 5b). To verify AT_1 receptor blockade, afferent arteriole diameter did not change when exposed to Ang II (10^{-9} mol L^{-1}) in either the control + Losartan (98 ± 1% of baseline diameter) or the LPS + Losartan (98 ± 1% of baseline diameter) groups. These data demonstrate that AT_1 receptor blockade decreases afferent arteriole vasoconstriction in LPS treated rats, but the impaired autoregulatory behaviour observed with this model of LPS treatment is independent of AT_1 receptor activation.

Scavenging superoxide with Tempol restored afferent arteriole autoregulatory behaviour in LPS treated rats

Increased ROS contribute to renal injury and impairment of renal autoregulatory behaviour in different models including normal kidneys, endotoxemia, high salt diet, and Ang II hypertension [11, 37, 49–53]. Animals were treated with LPS 4 hours prior to experiments. Acute superfusion of Tempol, a superoxide dismutase mimetic, did not alter the baseline diameter in the LPS group (Fig. 6a). Under these conditions, arteriole diameter increased to 112 ± 4% of baseline in the LPS + Tempol group (P<0.05, LPS vs. LPS + Tempol; Fig. 6b) when perfusion pressure was decreased from 100 to 65 mmHg. Subsequent increases in perfusion pressure to 170 mmHg in the LPS + Tempol group demonstrated pressure-induced vasoconstriction similar to control kidney (P<0.05, LPS vs. LPS + Tempol; Fig. 6b). These data indicate that ROS contributes importantly to afferent arteriole autoregulatory impairment during LPS treatment.

Blockade of TLR4 during acute LPS treatment prevented upregulation of renal vascular TLR4 protein expression

We examined TLR4 (≈100 kDa) protein expression in small intra-renal arteries by western blot analysis. Five groups (n=5/group) were prepared as described in the methods section, (control, LPS, LPS + IgG_{2a}, LPS + anti-TLR4, and LPS + LPS-RS) and used for harvesting small intra-renal arteries. Figure 7 illustrates the ratio of renal vascular TLR4 protein expression over β-actin. In the LPS group, renal vascular TLR4 protein (0.84 ± 0.13) was

significantly increased compared to control (0.41 ± 0.10; P<0.05). Antagonism of TLR4 with anti-TLR4 or LPS + LPS-RS prevented the increase of TLR4 protein in intra-renal arteries (0.43 ± 0.01 and 0.45 ± 0.16; P<0.05, respectively; Fig. 7b). Collectively, these data demonstrate that LPS increases renal vascular TLR4 protein expression and this increase can be prevented by inhibiting TLR4 activation during LPS treatment.

DISCUSSION

This study demonstrates that renal tubular and vascular smooth muscle cells express TLR4 in control and LPS treated animals and that tubular and vascular expression levels increase with acute LPS treatment. Both Losartan and/or LPS treatment significantly decrease SBP over the treatment period. Acute TLR4 activation with low-dose LPS treatment impairs afferent arteriole autoregulatory behaviour and this impairment is prevented by inhibition of TLR4 activation by either anti-TLR4 antibody or TLR4 blockade. Blockade of AT_1 receptors with Losartan, relaxed afferent arterioles in control and LPS treated rats, but did not prevent autoregulatory impairment during LPS treatment. Scavenging superoxide with Tempol, a superoxide dismutase mimetic, restores autoregulatory behaviour in afferent arterioles from LPS treated rats. These novel findings suggest that acute TLR4 activation, with a low dose of LPS, impairs renal microvascular autoregulatory function partially through a ROS-dependent mechanism.

Autoregulation is an intrinsic property of afferent arterioles that allows regulation of stable RBF and glomerular capillary pressure over a wide range of arterial pressures [1, 2]. Loss of renal autoregulatory efficiency can lead to inappropriate transmission of arterial pressure to the glomerular capillaries leading to glomerular sclerosis, CKD and renal failure. Renal autoregulatory behaviour is impaired in pathological conditions such as Ang II hypertension, high dietary salt, diabetes, DOCA-salt hypertension, and Dahl salt-sensitive hypertension [2, 4, 35, 36]. In the present study, acute 4-hour LPS treatment impaired afferent arteriole autoregulatory behaviour, but vasoconstrictor responses to KCl, NE, and P2 receptor agonists were normal compared to control arterioles. This argues that the contractile apparatus is functional in LPS treated rats but the signalling pathways that transduce changes in transmural pressure into appropriate changes in afferent arteriolar resistance are impaired.

The kidney utilizes the myogenic (~65%) and tubuloglomerular feedback (TGF; ~35%) mechanisms to accomplish whole kidney autoregulation of RBF, though the existence of a third mechanism has also been proposed [2, 54, 55]. Several studies determined the relative influence TGF plays on afferent arteriole autoregulatory behaviour [56–58]. Moore and Casellas demonstrated that TGF-dependent resistance adjustments of afferent arterioles were greatest near (21 ± 2 μm, or last 4% of arteriole length) the glomerular attachment, and that it was diminished by ~60% at a mid-afferent arteriole location (134 ± 6 μm from the glomeruli or last 27% of arteriole length) [56]. In the present study, afferent arterioles studies averaged 420 ± 14 μm in total length and diameters were measured near the midpoint of arteriole length (239 ± 9 μm or a distance of ~57% away from the glomeruli). Therefore, our measurements were made further away from the glomeruli than the Moore and Casellas

report [56] at a location where the myogenic influence would exceed the contribution of TGF, but participation of TGF cannot be completely ruled out.

Growing evidence indicates that activation of the immune system, immune cells, and inflammation play an important role in renal vascular, tubular, and renal hemodynamic dysfunction [59–61]. Renal inflammation contributes to reduced autoregulatory capability [35–37]. A major mechanism by which inflammatory processes begin is by activation of pattern recognition receptors and the family of TLRs. TLRs are expressed in human renal tissue especially in glomerular capillaries and renal tubules [18]. Tubular epithelia and mesangial cells express TLR1, TLR2, TLR3, TLR4, and TLR6 [62, 63]. TLR4 is found in the renal vasculature, glomeruli, and renal tubules [62]. Recent studies show that renal TLR4 protein expression increases in models of AKI and CKD [34, 63, 64]. For example, cortical TLR4 expression increases significantly 24 hours after cecal ligation and puncture, a murine model of sepsis [63]. Kim et al. reported increases in mRNA and protein expression of TLR4 in renal tubules after ischemia-reperfusion injury [65]. Although renal tubular TLR4 expression is upregulated in models of AKI, nothing is known about renal vascular TLR4 expression. We demonstrated that TLR4 is expressed in renal tubules by immunohistochemistry and immunofluorescence studies. TLR4 was also strongly expressed by renal vascular smooth muscle cells as shown by co-localization with α-smooth muscle actin, and expression levels increased following LPS treatment. These results demonstrate that TLR4 stimulation could enhance renal TLR4 expression initiating an immune response that contributes to reduced autoregulatory capability.

Evidence suggests that TLR4 contributes importantly to the progression of AKI and CKD [33, 66, 67], but the role of TLR4 on renal microvascular function is still unexplored. Dagher et al. showed that kidneys of TLR4$^{-/-}$ mice are protected against loss of renal function compared to WT controls in a model of AKI [68]. Furthermore, Souza et al. demonstrated that TLR4 deficient mice are protected from renal fibrosis and albuminuria during 5/6 nephrectomy with Ang II infusion [34]. Accordingly, we sought to determine the role of TLR4 in influencing renal microvascular function. Our studies revealed that inhibition of TLR4 with anti-TLR4 antibody, or competitive TLR4 antagonism with LPS-RS, preserved afferent arteriole autoregulatory behaviour during an LPS challenge. These data indicate that renal autoregulatory behaviour can be protected by prevention of TLR4 activation.

The mechanisms underlying the reduced blood pressure and increased renal vascular resistance during endotoxemia and sepsis are not completely understood. Studies have revealed the effects of endotoxemia and sepsis on the sympathetic nervous system [69, 70]. Schaller et al. reported that endotoxin treated rats (lipopolysaccharide *E. Coli* 0111:B4; 2 mg rat^{-1}; i.v.) exhibited a significant decrease in MAP compared to vehicle controls. Furthermore, plasma epinephrine, norepinephrine, renin activity, and vasopressin concentrations were significantly increased 90 minutes after endotoxin treatment, indicating an increase in sympathetic nervous system and renin angiotensin system activation during endotoxemia [69]. In a severe LPS (20 mg kg^{-1}; i.v; over 20 min) sepsis model, MAP decreased significantly (62 ± 7 mmHg), and was associated with elevated heart rate ($+ 20 \pm 6\%$) and renal sympathetic nerve activity ($+ 455 \pm 61\%$) three hours post LPS infusion [70].

Boffa et al. demonstrated that some of the renal vasoconstriction observed in endotoxemic mice is mediated by thromboxane A2 receptors [9]. Investigating whole kidney dynamic autoregulation using transfer function analyses, Nitescu et al. reported that endotoxemic rats (*E. Coli* 0127:B8; 7.5 mg kg^{-1}; i.p.; for 16 hours) exhibited markedly reduced GFR and RBF and impaired dynamic autoregulation arising from reduced TGF function but that the myogenic component appeared intact. In our study, we used a lower LPS dose and a shorter incubation period but found markedly impaired autoregulatory behaviour in a region of the arteriole where autoregulatory responses are largely myogenic. The reason for this discrepancy with the work of Nitescu [11] is unclear but may relate to the type or dose of LPS used.

Nitescu [11, 71] also reported that inhibition of AT_1 receptors with candesartan improved RBF and the TGF component of dynamic RBF autoregulation in the LPS rats, but did not normalize GFR [11, 71]. These studies suggest that there is a mechanistic link between AT_1 receptor activation and impaired renal microvascular dysfunction during endotoxemia. In the present study, we found that pre-treatment with Losartan, an AT_1 receptor blocker, significantly decreased SBP and increased baseline afferent arteriole diameter in control and LPS treated rats, ameliorating the LPS-induced increase in afferent arteriole vascular resistance. Interestingly, the autoregulatory impairment in the LPS + Losartan group was nearly identical to that with LPS alone, indicating that AT_1 receptor blockade did not restore autoregulatory capability. Prior studies from our laboratory have shown that Ang II (10^{-9} mol L^{-1}) reduces afferent arteriole diameter by approximately 19% [37, 39, 72–76]. In the present study, arterioles in Losartan treated kidneys were completely unresponsive to Ang II (10^{-9} mol L^{-1}) confirming effective AT_1 receptor blockade. Therefore, these data demonstrate that AT_1 receptor activation contributes to the increased afferent arteriole resistance during exposure to LPS, but does not contribute to autoregulatory dysfunction in LPS treated rats.

TLR4 activation is often associated with accumulation of ROS [32, 77]. Studies support interaction of TLR4 with NAD(P)H oxidase 4 in human embryonic kidney cells (HEK293T cells), in human aortic vascular smooth muscle cells, and in human mesangial cells [78–80]. Thus, there appears to be a close mechanistic relationship between TLR4 activation, NADPH oxidase activation and ROS generation. We found that acute exposure to Tempol restored pressure-mediated vasoconstriction in afferent arterioles from LPS treated rats that typically exhibit marked autoregulatory impairment, whereas, it had no detectable effect on autoregulation in normal kidneys [37]. These data suggest that accumulation of ROS, possibly superoxide, plays an important role in afferent arteriole autoregulatory impairment in the rat. Recent studies using isolated murine afferent arterioles by Lai et al. showed that increasing perfusion pressure led to increased superoxide production and enhanced myogenic reactivity, while treatment with Tempol, pegylated-SOD, or apocynin, all decreased ROS production [51]. The apparent discrepancy in the role of ROS in autoregulatory signalling could be explained by the two different methods used to study afferent arterioles. Lai et al. conducted experiments in mouse isolated afferent arterioles, while this study employed Sprague Dawley rats and the juxtamedullary nephron preparation where the afferent arterioles are maintained in their microenvironment within the kidney [51]. Alternatively, it could reflect differences in induction of oxidative signalling or the nature of the ROS

induced. In any case, ROS play an important role in autoregulatory signalling but further work is needed for the specific mechanisms and conditions to be resolved.

P2 receptors play an important role in afferent arteriole autoregulatory behaviour in normal and pathological conditions, such as high salt diet and hypertension [36, 81, 82]. P2 receptor function, specifically $P2X_1$, is critical for appropriate afferent arteriole pressure-mediated resistance adjustments [39]. In models of impaired afferent arteriole autoregulation, $P2X_1$ receptor reactivity is also impaired [42, 43, 83]. In the current study, we found that acute LPS treatment impairs afferent arteriole autoregulatory behaviour. Therefore, we hypothesized that $P2X_1$ receptor reactivity would also be impaired in acute LPS treated rats. LPS treatment for 4 hours did not significantly alter afferent arteriole responses to P2 receptor activation. Even though P2 receptor reactivity was relatively normal, afferent arterioles from LPS treated rats still exhibited reduced autoregulatory capability. Impaired renal autoregulation with normal P2 receptor reactivity is unusual compared to what is observed with longer (14 days) term high salt and Ang II hypertensive settings and may reflect the duration of the "insult" imposed [36, 43]. One study, reported normal afferent arteriole P2 receptor reactivity, with impaired autoregulatory behaviour during acute (~15–20 min) topical exposure to TGF-β [37]. Taken together, a potential explanation for the apparent disassociation between impaired autoregulation and P2 reactivity is that the 4-hour LPS exposure or short term exposure of TGF-β is too short to significantly impact P2 receptor trafficking and signalling. Another possible explanation is that during septic conditions where TLR4 would be stimulated, electron transport is compromised leading to a decrease in cellular ATP production [84, 85]. In that setting, the reduced autoregulatory performance observed could reflect a decrease in ATP available for extracellular release and stimulation of renal microvascular P2 receptors. This reduced ATP release could blunt autoregulatory responsiveness.

In conclusion, this study provides novel evidence that acute stimulation of TLR4 causes afferent arteriole autoregulatory dysfunction, in part through a ROS-dependent mechanism, but independent of P2 and AT_1 receptors. Inhibition of TLR4 signalling with anti-TLR4 antibody or LPS-RS, preserves renal autoregulatory behaviour. Overall, inhibition of TLR4 signalling and scavenging of ROS, during LPS treatment, confers significant protection of renal autoregulatory control. More work is needed to determine the mechanistic link between activation of TLR4, ROS accumulation, and impairment of renal microvascular function in renal pathologies.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (n=217; Charles River Laboratories, Raleigh, NC) weighing 345–420g were housed in institutional animal facilities in a 12–12 light-controlled room, with *ad libitum* access to drinking water and standard chow (PMI diet #: 5JL5; Lab Diets, St. Louis, Missouri, USA) All experiments were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals using procedures approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Alabama at Birmingham and Georgia Regents University – currently named Augusta University.

Expression of renal vascular TLR4 by immunofluorescence

Rats were anesthetized with pentobarbital (50 mg kg^{-1}; i.p.; Vortech Pharmaceuticals LTD, Dearborn, Michigan, USA) and the kidneys were perfused through the abdominal aorta. The kidneys were flushed with 5.2% bovine serum albumin (BSA) Tyrode's solution. The kidneys were excised, decapsulated, and sectioned longitudinally, placed in optimized cutting temperature (OCT) gel and frozen over dry ice. OCT frozen kidneys were stored at −80°C. Briefly, kidney sections (6 μm) were cut in a cryostat and mounted to positively charged microscope slides (Fisher Scientific), fixed with pre-cooled acetone, and then washed in 1x phosphate buffered saline (PBS). The kidney sections were incubated first with primary antibody for TLR4 (ab22048; 1:500; Abcam), followed by primary antibody for α-smooth muscle actin (1:1000) overnight at 4°C. The slides were rinsed with PBS and incubated with both the TSA-Cy3 donkey anti-mouse horse-radish peroxidase (HRP) (1:400; Invitrogen) and TSA-FITC (donkey anti-rabbit; 1:400; Invitrogen) secondary antibodies for 60 minutes and then Hoechst nuclear stain for 5 minutes at room temperature. Slides were quenched with 3% H_2O_2 (10 minutes), washed in 1x PBS, and blocked (PBS blocking buffer) in between each step. After a final rinse with PBS, sections were covered with 50% glycerol and coverslips were applied. Immunofluorescence images were captured using a Nikon A1R Confocal microscope with the Nikon Plan Flour 40x DIC N2 oil immersion objective. Images were analysed using the Nis Element 4.50 Imaging Software (Nikon) in cooperation with the UAB High Resolution Imaging Facility.

Systolic blood pressure (SBP) monitoring

Conscious SBP was measured on the kidney and blood donor rats using tail cuff plethysmography (IITC Life Science, Woodland Hills, CA) at time 0 and again every hour (1–4 hour), covering the 4-hour LPS treatment, until the kidneys and blood were harvested for study. In both Losartan treated groups (control + Losartan and LPS + Losartan), SBP was measured at time 0 and again every hour (1–5 hours), covering the Losartan and/or LPS treatment period.

In vitro blood-perfused juxtamedullary nephron preparation

Kidneys were prepared for blood perfused juxtamedullary nephron experiments as previously described [39, 86]. Briefly, two identically treated rats were anesthetized with pentobarbital (50 mg kg^{-1};i.p; Vortech Pharmaceuticals LTD, Dearborn, Michigan, USA) for each experiment. The right kidney from the kidney donor rat was cannulated via the superior mesenteric artery and continuously perfused with a Tyrode's solution containing 5.2% BSA (Calbiochem, La Jolla, CA).

Blood from both the kidney and blood donor rats was collected into heparinized syringes (500 IU) via a carotid artery catheter and processed by centrifugation. The plasma fraction was collected and erythrocytes were washed twice with saline. The plasma and washed erythrocytes were mixed to form a reconstituted blood perfusate (hematocrit ≈ 33%).

The perfused kidney was harvested and prepared for videomicroscopy [39, 86]. After completion of the dissection, the perfused kidney was visualized under a light microscope (Nikon Optiphot2-UD; Nikon, Tokyo, Japan) fitted with a Zeiss water-immersion objective

(40x) and superfused with Tyrode's buffer containing 1% BSA at 37°C. The perfusate was switched from Tyrode's solution containing 5.2% BSA to the reconstituted blood delivered from a pressurized reservoir continuously gassed with $95\% O_2/5\% CO_2$. Perfusion pressure was monitored using a pressure cannula positioned near the tip of the perfusion cannula and connected to a pressure transducer (Model TRN005; Kent Scientific Corporation, Torrington, CT). The focused image of the inner cortical surface and afferent arteriole was displayed on a video monitor and recorded on DVD for later analysis. Afferent arterioles averaged 420 ± 14 μm in total length and were monitored at a single site (239 ± 9 μm from the glomeruli or approximately 57% of total arteriole length; pooled data; n=84). Diameters were measured every 12 seconds using an image shearing monitor (Model 908; Vista Electronics, Valencia, CA). The mean arteriole diameter was calculated using all the diameter measurements obtained in the final 2 minutes of each treatment or pressure period.

Experimental protocols

After an equilibration period (10–15 minutes) with perfusion pressure held at 100 mmHg, the following experimental protocols were performed. Each protocol began with a 5-minute control period at 100 mmHg to establish a baseline afferent arteriole diameter.

Experiment 1: Effect of LPS on afferent arteriole autoregulatory behaviour—
Eight groups were studied (n=6/group): control, LPS (1 mg kg^{-1}; Lipopolysaccharide E. coli 055:B5; Sigma Aldrich, St. Louis, MO, USA), LPS (1 mg kg^{-1}) + TLR4 antagonist, LPS-RS, (5 mg; Invivogen, San Diego, CA, USA) LPS (1 mg kg^{-1}) + anti-TLR4 antibody (1 μg; sc-13591; Santa Cruz Biotechnology, Dallas, Texas, USA), LPS (1 mg kg^{-1}) + IgG$_{2a}$ antibody (1 μg; sc-69917; Santa Cruz Biotechnology) and LPS (1 mg kg^{-1}) + Tempol (10^{-3} mol L^{-1}; Sigma Aldrich). The LPS group received an intraperitoneal injection of a low dose of LPS 4 hours prior to kidney harvesting. The LPS + LPS-RS group was co-treated (i.p.) with LPS and LPS-RS 4 hours prior to experiments. The LPS + anti-TLR4 antibody group received the antibody treatment (i.p.) 12 hours prior to LPS dosing and the kidney was collected 4 hours after LPS treatment. The LPS + Tempol group received LPS (i.p.) 4 hours prior to experiments, and Tempol (10^{-3} mol L^{-1}) was added to the Tyrode's buffer superfusion solution approximately 15 minutes before assessment of autoregulatory capability.

In cases of endotoxemia or sepsis, Ang II levels reportedly increase in concert with the hypotensive decline in blood pressure [6, 69, 87]. To control for Ang II effects, we prepared two additional groups (control + Losartan (2 mg kg^{-1}; i.p.; Sigma Aldrich), and LPS (1 mg kg^{-1}; i.p.) + Losartan (2 mg kg^{-1}; i.p.) to compare with those above. The LPS + Losartan group received Losartan 1 hour prior to LPS treatment and the kidney was collected 4 hours after the LPS. Losartan (10 μmol L^{-1}) was added to the 5.2% BSA perfusion solution, 1% BSA superfusion solution, and the reconstituted blood perfusate. At the end of the autoregulatory protocol, Ang II (10^{-9} mol L^{-1}) was applied to the inner cortical surface to verify effective AT$_1$ receptor blockade.

Autoregulatory behaviour of afferent arterioles was assessed by measuring changes in lumenal diameter in response to step-wise changes in perfusion pressure. Autoregulatory behaviour was assessed by reducing perfusion pressure from 100 to 65 mmHg, and then

increasing perfusion pressure from 65 to 170 mmHg in 15 mmHg increments at 5 minute intervals.

Experiment 2: Afferent arteriole responses to potassium chloride (KCl) and NE—Voltage dependent calcium channels are essential for efficient autoregulation [41]. Therefore, the effect of LPS on the response to KCl was studied using afferent arterioles from control and LPS (1 mg kg^{-1}; i.p.) treated rats (n=4). Baseline afferent arteriole diameters were measured at 100 mmHg before the superfusion solution was changed to a similar solution containing 55 mmol L^{-1} KCl (substituted for NaCl). After 5 minutes of KCl exposure the superfusion solution was returned to 1% BSA for a 5-minute recovery period. Lumenal diameters were continuously monitored before, during, and after KCl treatment.

Adrenergic influences are important regulators of renal vascular resistance [75]. Accordingly, the effect of LPS on the response to NE, was studied in a separate group of (n=4) control and LPS (1 mg kg^{-1}; i.p.) treated kidneys. Following a 5-minute control period, arterioles were exposed to NE (100 nmol L^{-1}; Sigma Aldrich) for 5 minutes with renal perfusion pressure maintained at 100 mmHg. Five minutes later, the superfusion solution was returned to 1% BSA for a 5-minute recovery period.

Experiment 3: Afferent arteriole response to endogenous P2 receptor ligands—P2 receptors have been implicated in mediating autoregulatory changes in afferent arteriole diameter [42, 45]. Afferent arteriole concentration-response relationships to adenosine triphosphate (ATP; P2 agonist; Sigma Aldrich; n=6) beta-gamma-methylene ATP (β,γ-mATP; Selective P2X agonist; Sigma Aldrich; n=6), and uridine triphosphate (UTP; P2Y$_2$ agonist; Sigma Aldrich; n=6) were determined to establish P2 receptor reactivity in afferent arterioles from control and LPS treated kidneys [88]. Arterioles were exposed to ATP, β,γ-mATP, or UTP at concentrations of $10^{-8} - 10^{-4}$ mol L^{-1} while perfusion pressure set at 100 mmHg.

Expression of TLR4 in intra-renal arteries by western blot

Five groups were studied (n=5/group): control, LPS (1 mg kg^{-1}; i.p.), LPS + IgG$_{2a}$ (1 μg; i.p.), LPS + anti-TLR4 antibody (1 μg; i.p.), and LPS + LPS-RS (5 mg; i.p.). Rats were anesthetized with pentobarbital (50 mg kg^{-1}; i.p.) and both kidneys were perfused through the aorta with 5.2% BSA Tyrode's solution. The kidneys were removed, sectioned longitudinally, and placed in ice-cold physiologic salt solution (PSS). Intra-renal arteries were dissected from the inner cortex using a stereo microscope, snap frozen in liquid nitrogen, and stored at −80°C for protein isolation. The tissue was homogenized in RIPA lysis buffer with a protease inhibitor mini complete tablet (Roche Diagnostics, Indianapolis, IN USA) and 1mM phenylmethylsulfonyl fluoride (PMSF). The lysis buffer solution was centrifuged at 20,000xg for 12 minutes at 4°C and supernatant was aliquoted and stored at −80°C. The protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories Hercules, CA). Equal amounts of protein for each sample were denatured in SDS sample buffer at 95–100°C for 5 minutes, separated by electrophoresis on Bolt™ 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes, (Amersham™ Hybond ECL GE Healthcare Life Science). Membranes were blocked in 5%

nonfat dry milk in TBS-T 0.1% Tween 20 at room temperature for 60 minutes. Membranes were incubated for 16 hours with mouse anti-TLR-4 primary antibody (ab22048; 1:5000; Abcam) at 4°C. The blots were washed and incubated again with goat anti-mouse (1:5000; Cell Signalling, Danvers, MA) secondary antibody conjugated with horseradish peroxidase at room temperature for 60 minutes. The membrane was washed and visualized by enhanced chemiluminescence (Clarity Western ECL substrate), and images were developed after exposure to X-ray film. The same blots were stripped, washed and re-probed with monoclonal anti-β-actin (1:10,000, Sigma Aldrich) as a loading control.

Expression of TLR4 by immunohistochemistry

Rats were anesthetized with pentobarbital (50 mg kg^{-1}; i.p.) and the kidneys were perfused through the abdominal aorta. The kidneys were flushed with 5.2% BSA Tyrode's solution. The kidneys were excised, decapsulated, and fixed in 10% neutral buffered formalin for 24–48 hours at room temperature. The fixed kidneys were embedded in paraffin, cut into 5 μm sections and processed for immunohistochemistry. Briefly, the sections were deparaffinised in Citrisolv (Fisher Scientific), rehydrated, and placed in 10 mmol L^{-1} citrate buffer, and placed in a rice steamer for 30 minutes. After cooling (60 min.), sections were quenched in 3% H_2O_2 for 10 min and rinsed in PBS. The sections were blocked in PBS blocking buffer (0.1g BSA + 0.2% non-fat dry milk + 0.3 ml Triton X-100+100 ml 1X PBS) for 30 minutes at room temperature, and incubated with primary antibody for TLR4 (Abcam; ab22048; 1:2000) at 4°C in a humidity chamber overnight. Slides were washed in PBS and incubated with secondary antibody (SuperPicTure Polymer Detection; Invitrogen-879163) for 60 minutes at room temperature. 3,3′-Diaminobenzidine (DAB) was added to the sections and incubated for 6 minutes, rinsed in dH_2O, counterstained, and covered with Cytoseal60. Prepared kidney sections were evaluated for TLR4 staining using an Olympus BX43 microscope with an Olympus DP73 camera.

Statistical analysis

Data are expressed as means ± SEM. Within-group analysis was conducted using one-way ANOVA for repeated measures with post-hoc analysis using Dunnett's multiple comparison test. Significant differences between groups were determined using one-way ANOVA and Dunnett's multiple comparison test or Student's t-test where appropriate. P values <0.05 were considered to indicate significant differences.

Acknowledgments

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Physiological Relevance

Immune system activation and inflammation are risk factors for multiple renal pathologies such as hypertension, high dietary salt, diabetes and ischemia-reperfusion injury. In these pathologies, damaged tissues can release damage-associated molecular patterns that in turn can activate TLR4. From this study, stimulation of TLR4 impairs autoregulatory efficiency that can lead to inappropriate transmission of arterial pressure downstream to the glomerulus. Activation of the immune system and inflammation, through tissue damage, could provide a mechanistic link between renal pathology and impaired kidney function. Therefore, resolving the mechanisms by which immune system activation and inflammation facilitate the loss of renal autoregulatory function could provide novel therapeutic targets for the prevention of or intervention in renal injury.
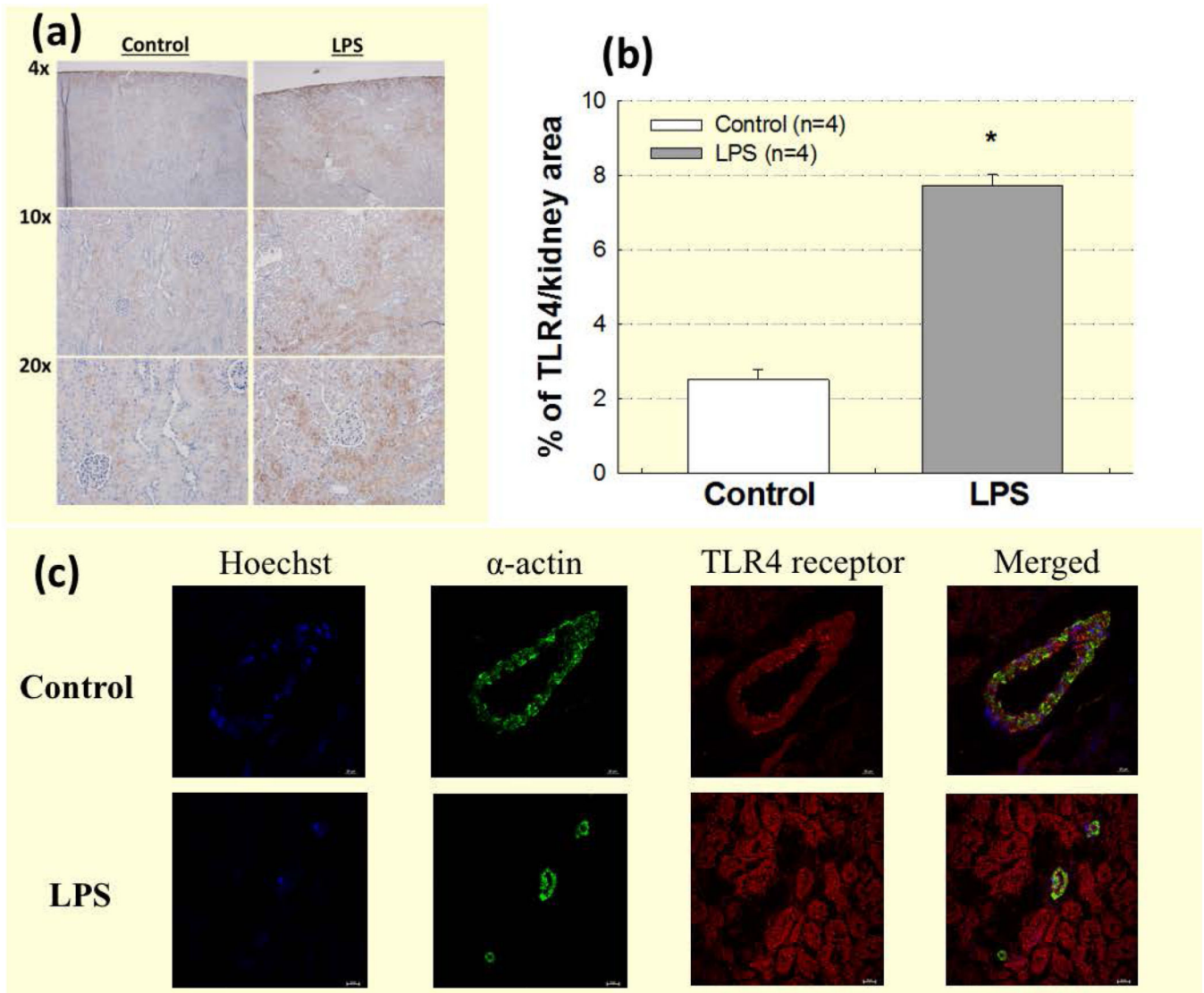
Figure 1. TLR4 is expressed in renal vascular smooth muscle cells and LPS treatment increases renal TLR4 expression

TLR4 is expressed in renal vascular smooth muscle cells, and LPS treatment increases renal TLR4 expression. (a): DAB (TLR4) staining of kidneys from control (left panels) and LPS (1 mg kg^{-1}; i.p.; right panels) treated groups. (b): Data are expressed as a percentage of total kidney area stained for TLR4 from control (white bars) and LPS treatment (grey bars). Each bar represents the mean ± SE. n=4 per group. (c): Immunofluorescence staining of TLR4 in OCT frozen whole kidney sections. Representative images show nuclear staining (blue; Hoechst), vascular smooth muscle α-actin (green), TLR4 (red), and co-localization of TLR4 and vascular smooth α-actin (yellow). Images were taken at 40x magnification. Bar = 20 μm. * P<0.05 vs. control TLR4 expression.
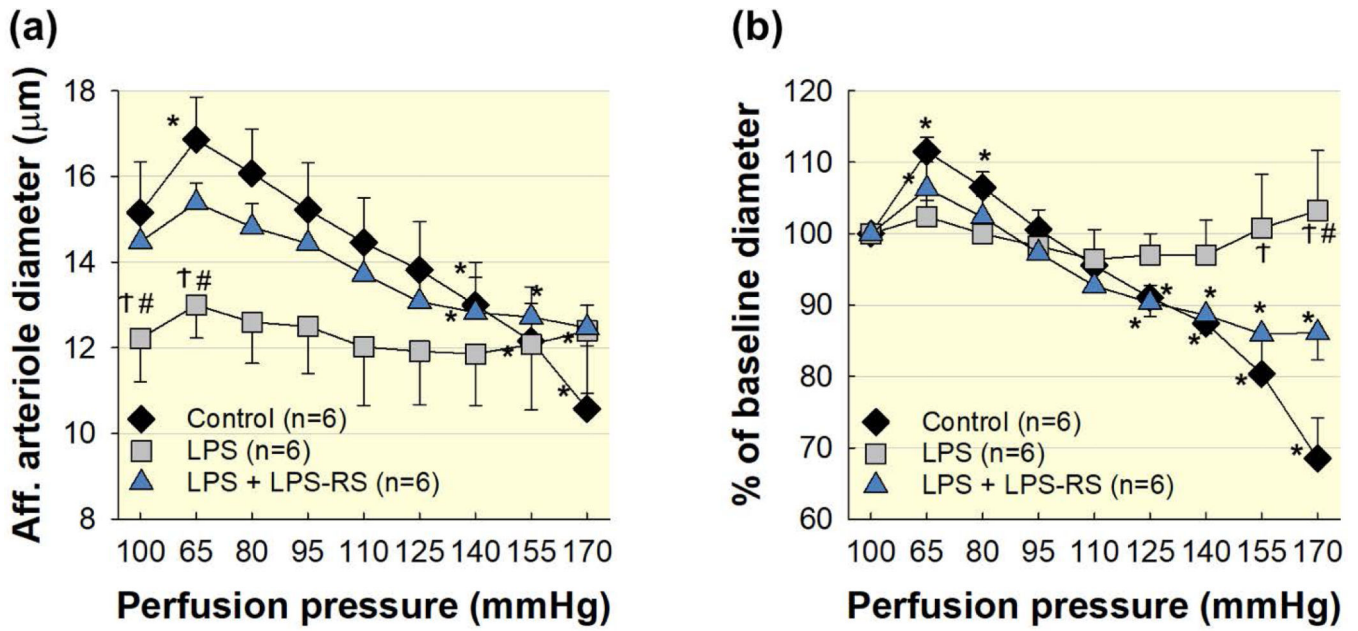
Figure 2. TLR4 blockade with LPS-RS preserves autoregulatory behaviour
Co-treatment with the TLR4 antagonist, LPS-RS, preserves afferent arteriole autoregulatory behaviour in LPS treated kidneys. (a): Effect of renal perfusion pressure changes on afferent arteriole diameter in kidneys from control (black diamonds), LPS (1 mg kg^{-1}; i.p.; grey squares), and LPS + LPS-RS (5 mg; i.p.; blue triangles) treated groups. (b): Data are expressed as a percent of the baseline diameter at 100 mmHg. Each data point represents the mean ± SE. n=6 per group. * P<0.05 vs. baseline diameter in the same group. † P<0.05 vs. control group for the same perfusion pressure. # P<0.05 vs. LPS-RS for the same perfusion pressure.
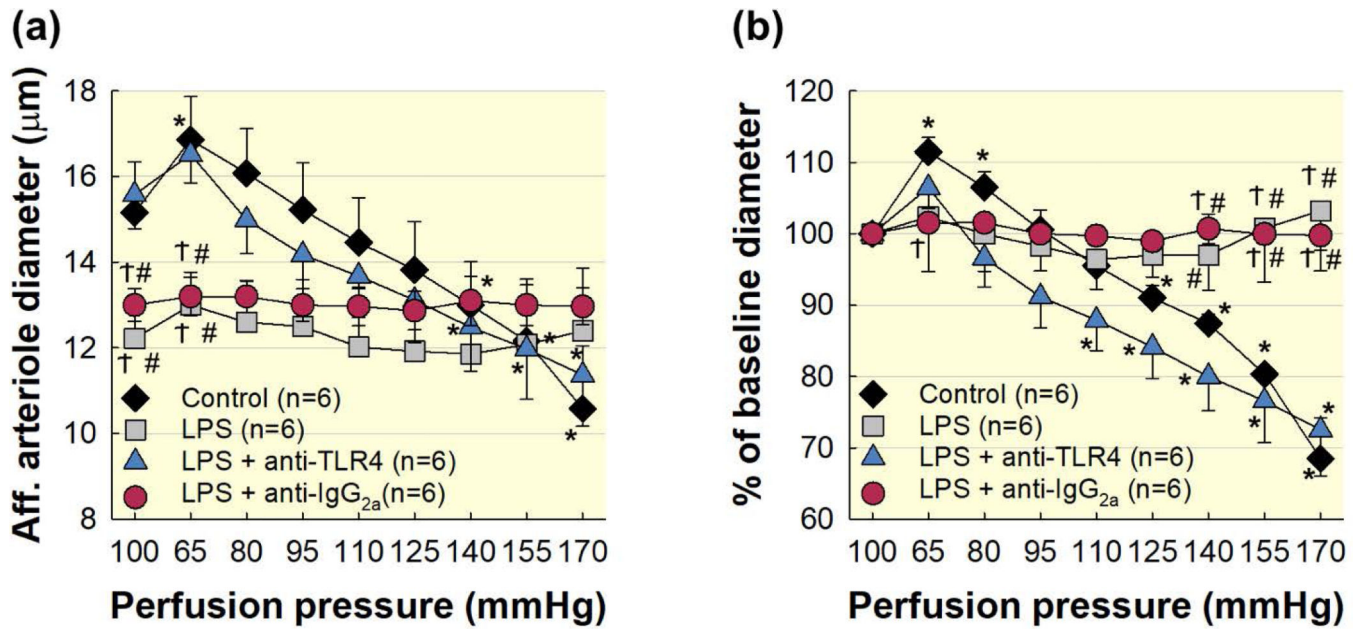
Figure 3. Pre-treatment with anti-TLR4 antibody preserved afferent arteriole autoregulatory behaviour during acute LPS treatment

Pre-treatment with anti-TLR4 antibody preserves afferent arteriole autoregulatory behaviour in kidneys from LPS treated rats. (a): Effect of renal perfusion pressure changes on afferent arteriole in kidneys from control (black diamonds), LPS (1 mg kg^{-1}; grey squares), LPS + anti-TLR4 antibody (1 µg; blue triangles), and LPS + anti-IgG2a antibody (red circles). (b): Data are expressed as a percent of the baseline diameter at 100 mmHg. Each data point represents the mean ± SE. n=6 per group. * $P<0.05$ vs. baseline diameter in the same group. † $P<0.05$ vs. control group for the same perfusion pressure. # $P<0.05$ vs. anti-TLR4 antibody for the same perfusion pressure.
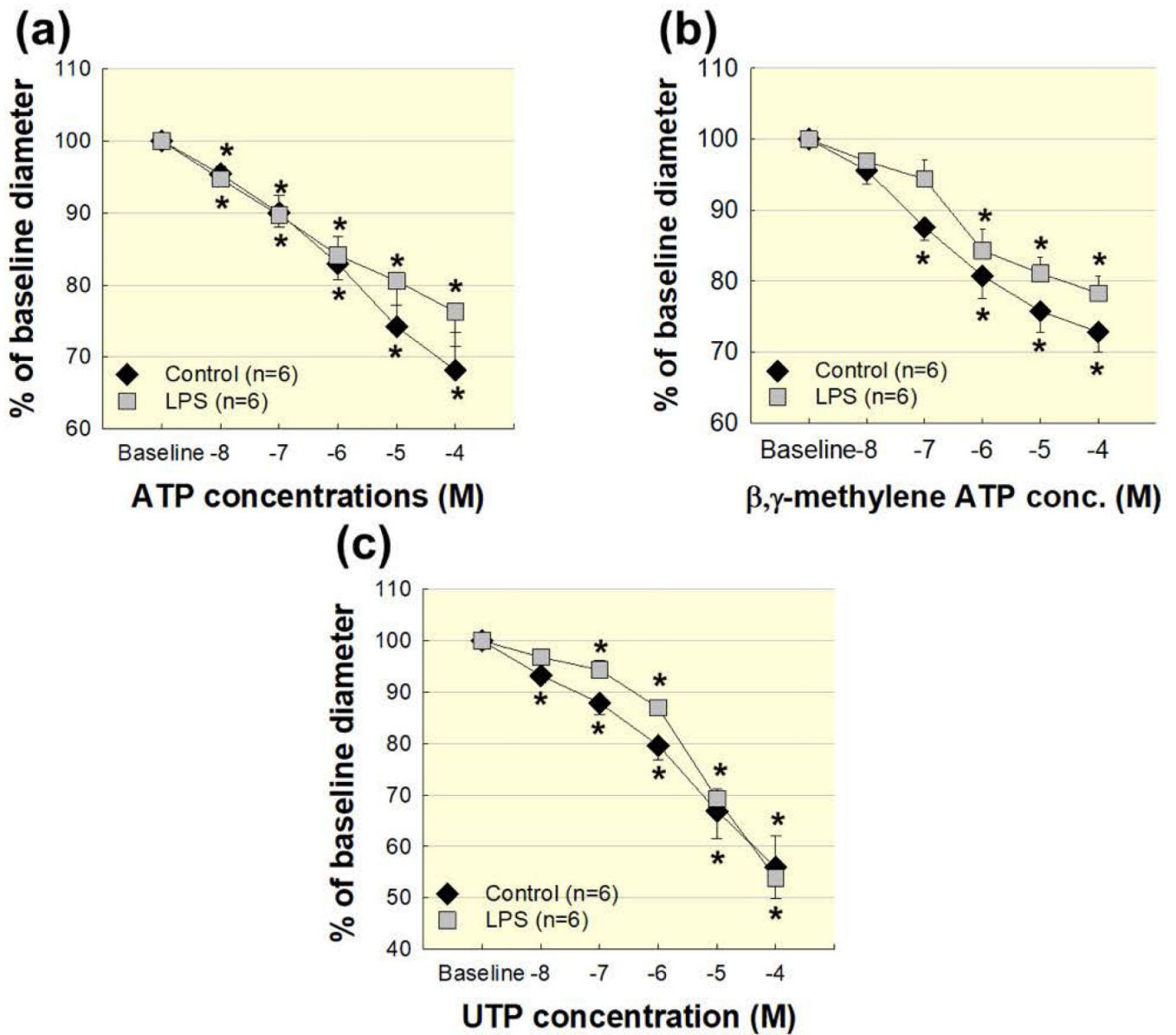
Figure 4. Afferent arteriole reactivity to ATP, β,γ-mATP, and UTP are normal in acute LPS treated rats

Afferent arteriole responses to ATP, β,γ-mATP, and UTP in acute LPS treated rats. (a): Afferent arteriole response to superfusion of ATP was assessed in control (black diamonds) and LPS (1 mg kg^{-1}; i.p.; grey squares). (b): Afferent arteriole response to superfusion of β,γ-mATP was assessed in control (black diamonds) and LPS (grey squares). (c): Afferent arteriole response to superfusion of UTP was assessed in control (black diamonds) and LPS (grey squares). Data are expressed as a percent of the baseline diameter at 100 mmHg. Each data point represents the mean ± SE. n=6 per group. * P<0.05 vs. baseline diameter in the same group.
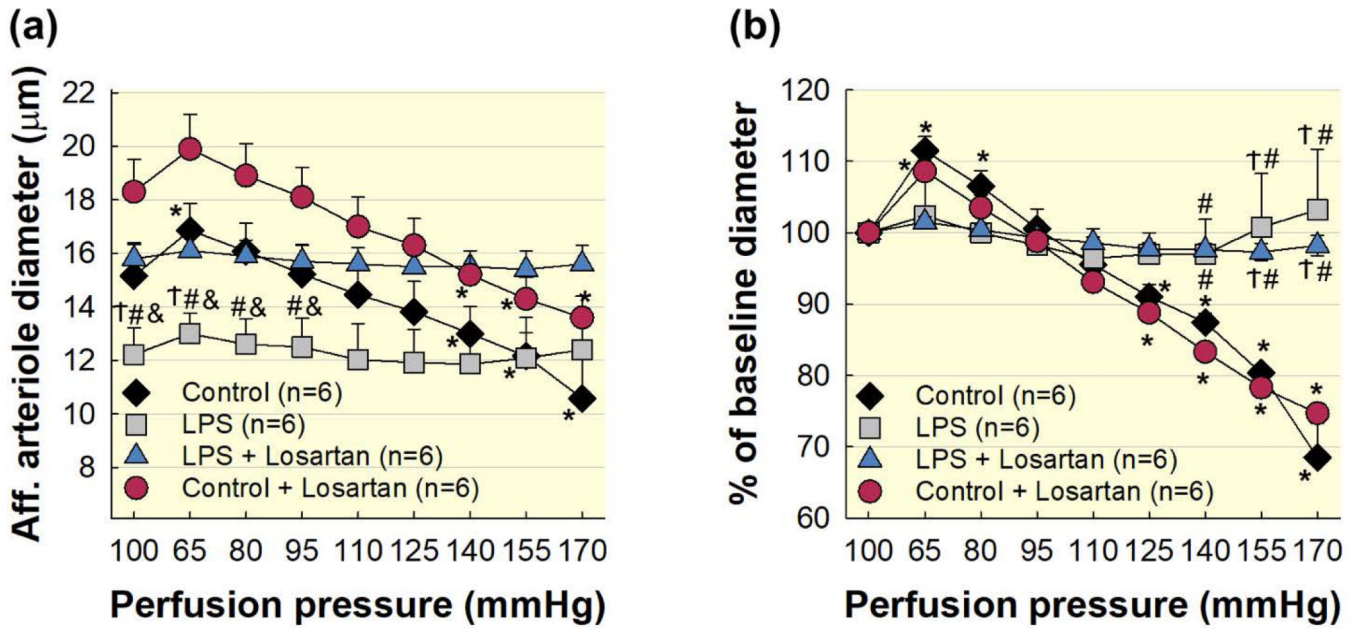
Figure 5. Impairment of afferent arteriole autoregulatory behaviour is independent of AT$_1$ receptor activation during acute LPS treatment

(a): Effect of renal perfusion pressure changes on afferent arteriole diameter in control (black diamonds), LPS (1 mg kg^{-1}; i.p.; grey squares), LPS + Losartan (2 mg kg^{-1}; i.p.; blue triangles), and control + Losartan (red circles). (b): Data are expressed as a percent of the baseline diameter at 100 mmHg during the equilibration period. Control and LPS data are included from Figure 2 for reference. Each data point represents the mean ± SE. n=6 per group. * P<0.05 vs. baseline diameter in the same group. † P<0.05 vs. control for the same perfusion pressure. # P<0.05 vs. control + Losartan for the same perfusion pressure. & P<0.05 vs. LPS + Losartan for the same perfusion pressure.
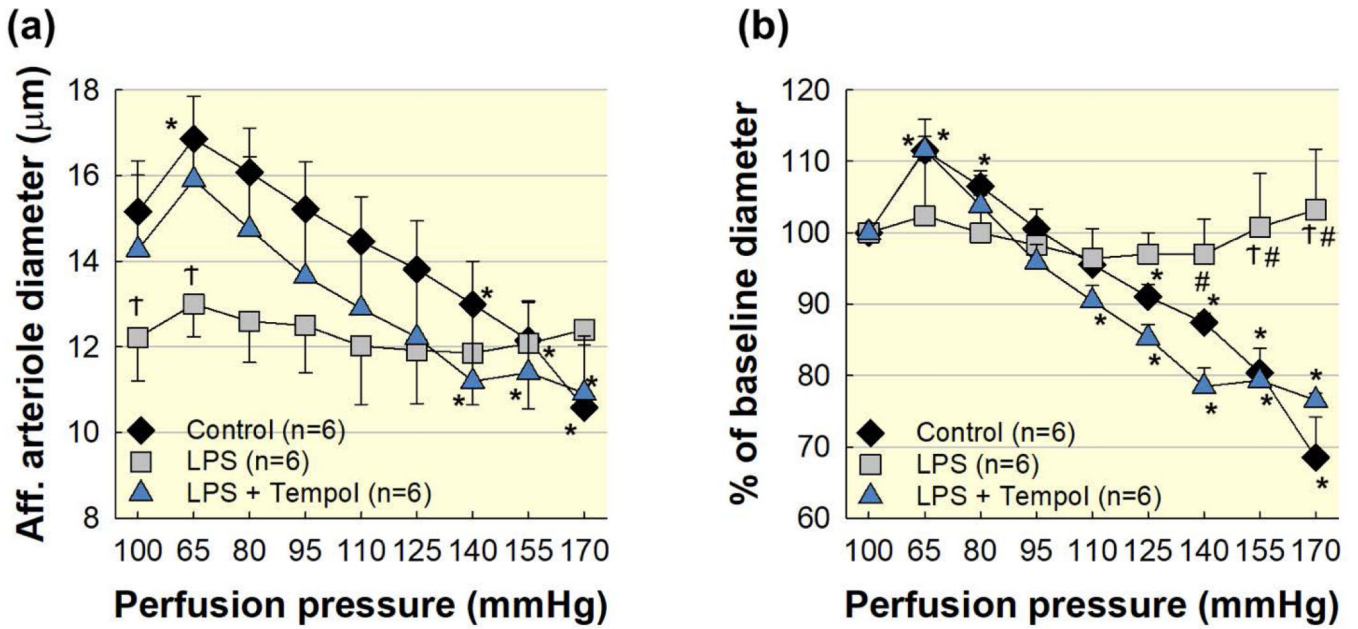
Figure 6. Acute superfusion of Tempol restores afferent arteriole autoregulatory behaviour in LPS treated rats

Acute superfusion of Tempol (10^{-3} mol L^{-1}) restores afferent arteriole autoregulatory behaviour in LPS treated kidneys. Control and LPS groups are the same as shown in Figure 2. (a): Effect of renal perfusion pressure changes on afferent arteriole diameter in control (black diamonds), LPS (1 mg kg^{-1}; i.p.; grey squares), and LPS + Tempol (blue triangles) kidneys. (b): Data are expressed as a percent of the baseline diameter at 100 mmHg. Each data point represents the mean ± SE. n=6 per group. * $P<0.05$ vs. baseline diameter in the same group. † $P<0.05$ vs. control group for the same perfusion pressure. # $P<0.05$ vs. Tempol for the same perfusion pressure.
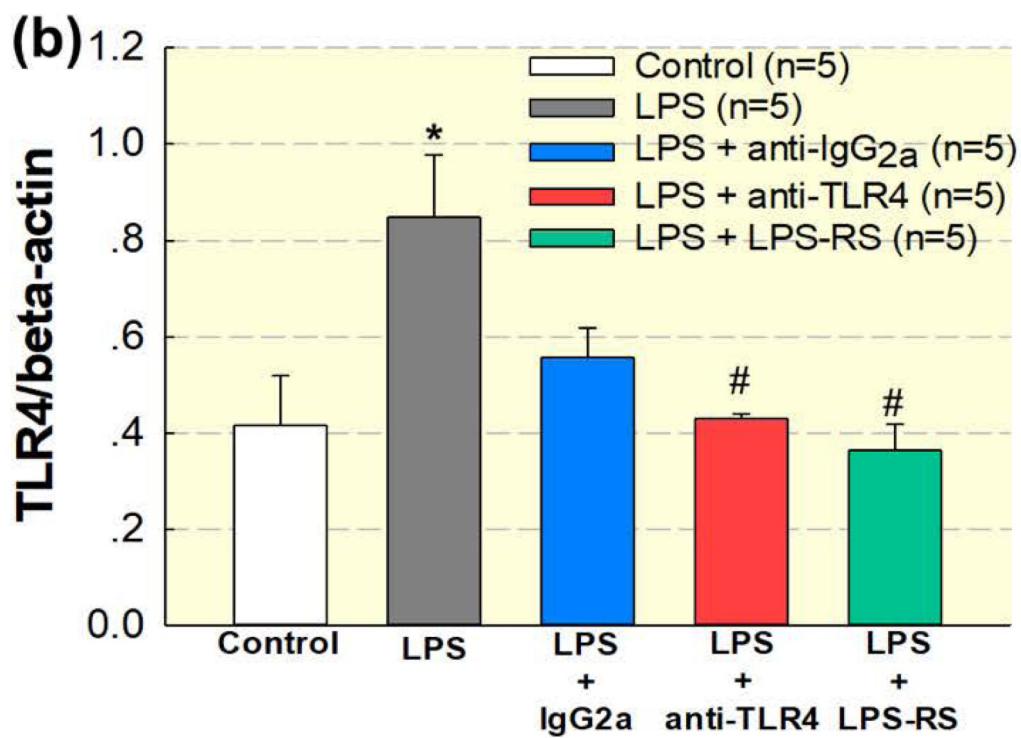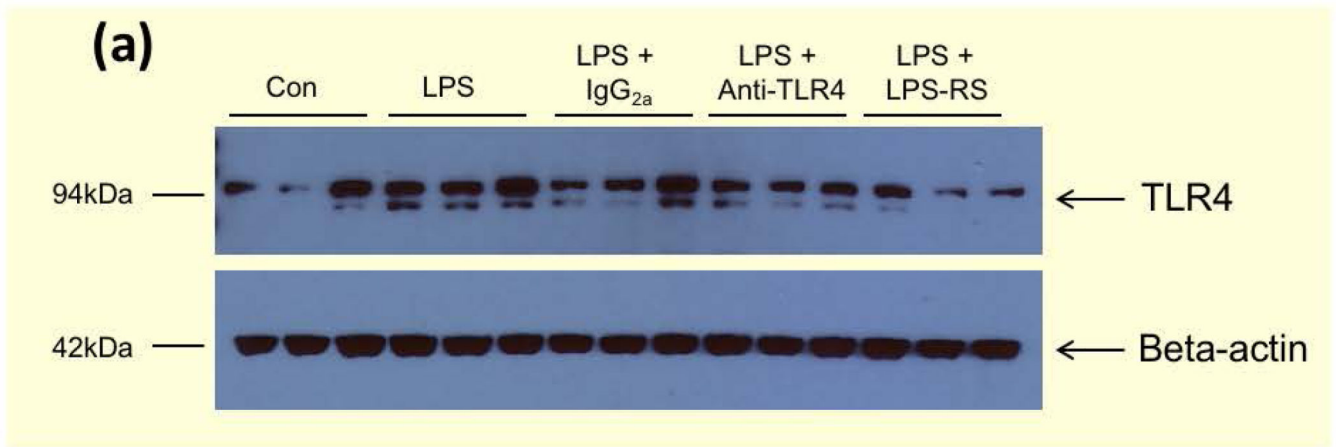
Figure 7. Blockade of TLR4 during LPS treatment prevents upregulation of renal vascular TLR4 protein expression

Treatment with anti-TLR4 antibody (1 μg; i.p.) or LPS-RS (5 mg i.p.) decreases TLR4 protein expression in small intra-renal arteries in LPS (1 mg kg^{-1}; i.p.) treated rats. (a): Representative image of typical gel. (b): Densitometry data normalized against β-actin. Each bar represents the mean ± SE. n=5 per group. * P<0.05 vs. control TLR4 expression. # P<0.05 vs. LPS TLR4 expression.

Table 1

The effect of acute LPS treatment on systolic blood pressure.

Treatment Group	Body Weight (g)	Baseline SBP (mmHg)	Hour 1 (mmHg)	Hour 2 (mmHg)	Hour 3 (mmHg)	Hour 4 (mmHg)
Control	372 ± 8	136 ± 2	131 ± 2	134 ± 2	134 ± 2	132 ± 2
LPS	365 ± 7	132 ± 1	$113 \pm 2 *†$	$102 \pm 2 *†$	$103 \pm 2 *†$	$109 \pm 1 *†$
LPS		−	+	+	+	+

*
P<0.05 vs. baseline SBP.

†
P<0.05 vs. control for same time point.

Table 2

The effect of Losartan treatment on systolic blood pressure in control and acute LPS treated rats

Treatment group	Body Weight (g)	Baseline SBP (mmHg)	Hour 1 (mmHg)	Hour 2 (mmHg)	Hour 3 (mmHg)	Hour 4 (mmHg)	Hour 5 (mmHg)
Control + Losartan	348 ± 8	136 ± 1	$124 \pm 1^*$	$124 \pm 1^*$	$127 \pm 1^*$	$124 \pm 1^*$	$124 \pm 1^*$
LPS + Losartan	356 ± 5	136 ± 1	$126 \pm 1^*$	$110 \pm 1^{*\dagger}$	$109 \pm 1^{*\dagger}$	$111 \pm 1^{*\dagger}$	$112 \pm 1^{*\dagger}$
Losartan		–	+	+	+	+	+
LPS		–	–	+	+	+	+

*
P<0.05 vs. baseline SBP.

$\dagger$P<0.05 vs. control + Losartan for the same time point.