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Differential Regulation of *Toll-Like Receptor 4* Gene Expression in Renal Cells by Angiotensin II: Dependency on AP1 and PU.1 Transcriptional Sites

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Key Words
Angiotensin II · Toll-like receptor 4 · Inflammation · Transcriptional activity, TLR4 promoter

Abstract
Background: Toll-like receptor 4 (TLR4) is involved in the sensing of lipopolysaccharide and, therefore, plays a central role in innate immune responses to Gram-negative bacteria. Interestingly, TLR4 expression occurs within the kidney. We have previously demonstrated that angiotensin II (ANG II) upregulates TLR4 expression on mesangial cells. However, the factors controlling transcriptional activation of the *Tlr4* gene in mesangial cells are not known, and the specificity of this response for other renal cells is unclear. ***Methods:*** Cultured murine proximal tubular cells (mouse cortical tubule cell line; MCT cells), murine mesangial cells (MMCs), and murine podocytes were treated with ANG II. The expression of ANG II receptor mRNA and TLR4 mRNA and protein was determined by polymerase chain reaction and Western blotting. The transcriptional activity of wild-type and mutant mouse TLR4 promoter reporter constructs was determined upon transient transfection of the three cell types. ***Results:*** Although MMCs, podocytes, and syngeneic proximal MCT cells similarly expressed ANG II receptors, ANG II stimulated TLR4 mRNA and protein expression in MMCs and podocytes only. A mouse TLR4 promoter construct (–518/+129), previously shown to contain all important transcriptional regulatory elements in various cell types, was activated by ANG II in MMCs and podocytes, but not in MCT cells. Mutation of a proximal PU.1-binding consensus site or an AP1 site abolished ANG-II-mediated transcriptional activation of the TLR4 promoter. Finally, basal transcription of the *Tlr4* gene depended in all three cell lines on an intact AP1 site and additionally on the proximal PU.1 site in MMCs. ***Conclusions:*** ANG II stimulates TLR4 transcription through AP1 and PU.1 sites in a cell-specific manner. Since the intrarenal ANG II concentrations are enhanced in many pathophysiological situations, ANG-II-stimulated transcription of TLR4 on MMCs and podocytes may contribute to renal inflammation.

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Introduction

Innate immunity and adaptive immunity are complementary mechanisms of defense against microbial threats. The adaptive immune system, being evolutionarily more recent and having the capacity for selectivity, adaptation, and memory, has arguably been regarded as more sophisticated and potent than the innate immune system. Yet, over the last years, the innate immune system has been

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ascribed an essential role in shaping adaptive immune responses. The sensing of microorganisms by innate immune cells relies on the expression of pattern recognition molecules, among which members of the Toll-like receptor (TLR) family play a particularly important role. The TLR family consists of at least 13 members in mammals. TLRs are widely distributed on the cell surface or inside cells in order to recognize and respond to a vast array of molecules such as lipids, proteins, and nucleic acids derived from pathogens. For example, TLRs have been implicated in the sensing of bacterial lipopolysaccharide and lipoproteins, viral RNA, and bacterial and viral unmethylated cytosine-guanosine dinucleotides containing DNA as well as various microbial proteins [1, 2]. The activation of signaling pathways after microbial product recognition by TLRs engages several adaptors, transducers, and transcription factors, resulting in the expression of inflammatory and immune-system-related genes.

In the kidney, TLRs are involved in many, if not all, types of renal inflammation [2]. We have previously found in vitro and in vivo that angiotensin II (ANG II) induces the expression of TLR4, the signal transducing molecule of the lipopolysaccharide receptor complex, by mesangial cells [3]. This ANG-II-mediated upregulation of TLR4 may be important for various inflammatory renal diseases such as chronic glomerulonephritis. In addition to mesangial cells, TLR4 is expressed by other renal cells [2]. The aim of the present study was to test whether ANG II modulates the TLR4 expression in murine mesangial cells, proximal tubular cells, and podocytes and to gain insight into cell-type-specific transcriptional activation of the *Tlr4* gene.

Materials and Methods

Reagents

Cell culture medium and fetal calf serum were from Gibco-BRL (Eggenstein, Germany). ANG II and interferon gamma were purchased from Sigma (Deisenhofen, Germany). The antimouse TLR4 polyclonal antibody was from Imgenex (Hamburg, Germany). Other antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). Lipofectamine 2000 and enzymes were purchased from Invitrogen (Karlsruhe, Germany). Luciferase assay reagent was from Promega (Mannheim, Germany) and β-galactosidase detection reagent from BD Biosciences (Heidelberg, Germany). All other chemicals were from Sigma.

Cell Culture

Mouse proximal tubular cells (mouse cortical tubule cell line; MCT cells) and mouse mesangial cells (MMCs) are cell lines originally derived from SJL mice [4–6]. Both cell lines are well characterized and exhibit many structural and functional features of their original tissue. Cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum in 5% CO_2 at 37°C. The cells were passaged every 4–5 days. Conditionally immortalized mouse podocytes were cultured as previously described [7]. They were maintained in RPMI 1640 medium with 10% fetal calf serum. Podocytes were cultivated at 33°C on type I collagen with 10 U/ml of recombinant interferon gamma. For induction of differentiation, the podocytes were cultured at 37°C without interferon gamma [7]. Differentiated podocytes were positive for synaptopodin by immunocytochemistry. All stimulation experiments were performed in differentiated podocytes.

Table 1. Primer sequences and annealing temperatures

Primer		5′–>3′ sequence	Temperature, °C
AT1a	forward	tcaaaagaattcaagatgact	56
	reverse	tccatataagagtatagctggtaag	
AT1b	forward	tgggaatatttggaaacagtttggt	58
	reverse	cagagtatagctggtgagaataat	
AT2	forward	tcctgttctctactacatgatttttg	61
	reverse	agccataatacaagcattcacac	
TLR4	forward	agtgggtcaaggaacagaagca	60
	reverse	ctttaccagctcatttctcacc	
β-Actin	forward	gatgatgcagataatgtttgaaac	60
	reverse	gagcaatgatcttaatcttcattgtg	
GAPDH	forward	accacagtccatgccatcac	66
	reverse	tccaccaccctgttgctgta	

Isolation of RNA, Reverse Transcription, and Semiquantitative and Real-Time PCR

Total RNA was isolated from 1×10^7 podocytes, MMCs, and MCTs cultured for 24 h with or without 10^{-7} M ANG II, using the RNAeasy kit (Qiagen, Hilden, Germany). cDNA was generated from 2 μg total RNA using the M-MLV reverse transcriptase kit (Qiagen). Of the resulting 50 μl of cDNA, 2 μl was subjected to PCR amplification using gene-specific primers (table 1). PCR amplifications were performed as follows: 95°C for 5 min followed by 30–35 cycles consisting of 95°C for 45 s, annealing temperature (as indicated in table 1) for 45 s, 72°C for 45 s, and an additional elongation step at 72°C for 5 min. Mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin was amplified to control the cDNA quantity. A negative control without cDNA was also performed.

Real-time PCR was performed in a final volume of 20 μl containing 1 μl of cDNA, 0.5 μM of each sense and antisense primer, and 4 μl master mix of the Lightcycler FastStart DNA Master-PLUS SYBR Green I kit (Roche Applied Science, Mannheim, Germany). Real-time PCR reactions were performed on a Mastercycler ep realplex (Eppendorf, Hamburg, Germany), according to the manufacturer's protocol. The expression of TLR4 and GAPDH mRNA was determined simultaneously in two parallel reactions in a 96-well plate. The measurements for TLR4 and GAPDH mRNAs were set up as subassays. The threshold cycle

represents the PCR cycle at which an increase of the reporter fluorescence above the baseline is first detected. The relative quantification analysis module is used to compare the expression levels of the target gene among differently treated samples. The expression levels are calculated using the ΔCT (threshold cycle) method. Real-time PCR experiments were independently performed nine times for each cell line and condition.

Western Blot Analyses

The cells were incubated with 10^{-7} M ANG II for 10 min or 24 h. After washing in ice-cold phosphate-buffered saline, the cells were lysed on ice in 150 μl of a buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 2.5 mM $MgCl_2$, 1% Triton X-100 supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, and protease inhibitors (Boehringer Mannheim, Mannheim, Germany). The protein content was determined by a modification of the Lowry method. Thirty micrograms of protein was run through 10% SDS-PAGE and transferred onto PVDF membranes by semidry Western blotting, as described elsewhere [6]. The membrane was blocked for 1 h in 5% nonfat milk in Tris-borate salt buffer containing 0.05% Tween 20. To assess the TLR4 expression, the membrane was incubated for 1 h with a polyclonal antibody against mouse TLR4 diluted 1:500 in 5% bovine serum albumin-Tris-borate salt, washed with Tris-borate salt/Tween 20, and incubated for 1 h with a horseradish peroxidase conjugated goat antirabbit secondary antibody (1:500). Signals were revealed using the ECL Western blotting system (Amersham, Munich, Germany). To control for variations in protein loading and transfer, the membranes were washed for 30 min in phosphate-buffered saline containing 0.1% Tween 20 and probed using an antibody specific for vinculin. The exposed films were analyzed by densitometry. TRL4 signals were normalized to vinculin signals and expressed relative to the ratio, set at 1.0, obtained from cells cultured without ANG II. Western blots were independently repeated four times.

Transient Transfection and Reporter Gene Assays

The mouse TLR4 promoter was cloned in the pGL3-basic vector. Wild-type and mutant constructs have been previously described in detail [8]. The cells were transfected with 8 μg of different mouse TLR4 promoter luciferase reporter vectors together with 0.2 μg of the pSV-β-galactosidase vector using Lipofectamine 2000. After transfection, the medium was changed into serum-free Dulbecco's modified Eagle's medium and the incubation prolonged for 24 h with or without 10^{-7} M ANG II. The cells were washed three times in phosphate-buffered saline and lysed. The protein concentration of the lysates was determined by the Bradford assay, and equal amounts of each lysate (usually 20 μl) were mixed in 96-well plates, (1) with 100 μl of luciferase assay reagent for the detection of firefly luciferase activity followed by immediate measurement for 10 s using a LUMIstar Galaxy luminometer (BMG Labtech, Offenburg, Germany), and (2) with 100 μl of luminescent β-galactosidase detection reagent for 60 min at room temperature before measurement of the β-galactosidase activity on a luminometer under the above conditions. The ratio between luciferase and β-galactosidase activities was calculated. The relative activity of the wild-type –518 TLR4 promoter in cells cultured without ANG II was set up as 1.0. Results are representative of at least three independent experiments performed in triplicate.

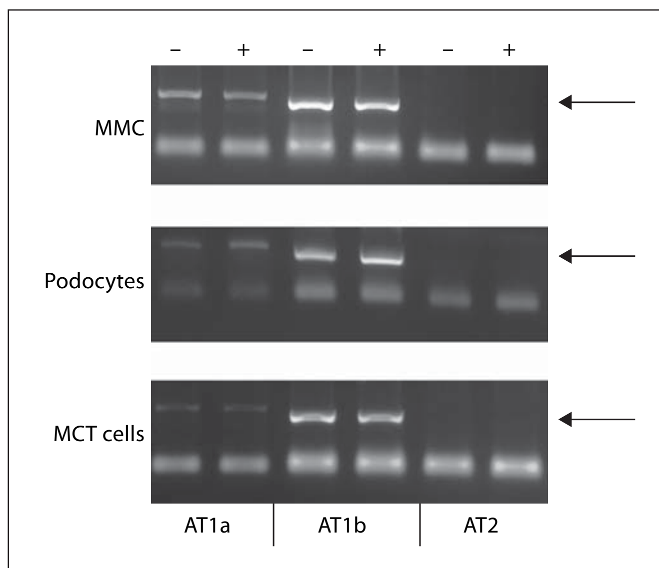

Fig. 1. Real-time PCR analysis of AT1a, AT1b, and AT2 ANG II receptor subtypes in MMCs, podocytes, and MCT cells. All three cell types expressed AT1a and AT1b receptor mRNAs. No AT2 receptor transcripts could be detected. ANG II (10^{-7} M for 24 h, +; – = no ANG II) did not modulate AT1a and AT1b receptor mRNA levels. Amplification of the housekeeping gene GAPDH showed equal concentrations of all cDNAs (data not shown). Results are representative of three independent experiments.

Statistics

All data are presented as mean values ± SEM. Statistical significance between different groups was first tested with the nonparametric Kruskal-Wallis test using SPSS version 14. Individual groups were subsequently tested using the Wilcoxon-Mann-Whitney test. $p < 0.05$ was considered statistically significant.

Results

MMCs, Podocytes, and MCT Cells Express AT1a and AT1b Receptor mRNA

First, we demonstrated that MMCs, podocytes, and MCT cells expressed AT1a and AT1b receptor transcripts at comparable levels (fig. 1). This represented a prerequisite necessary for further investigations comparing the effects of ANG II on the *Tlr4* gene expression in the different cell types. In addition, MMCs, podocytes, and MCT cells did not express the AT2 receptor transcript (fig. 1). Finally, treatment with 10^{-7} M ANG II for 24 h did not modulate the AT1 receptor mRNA expression in either cell line (fig. 1).

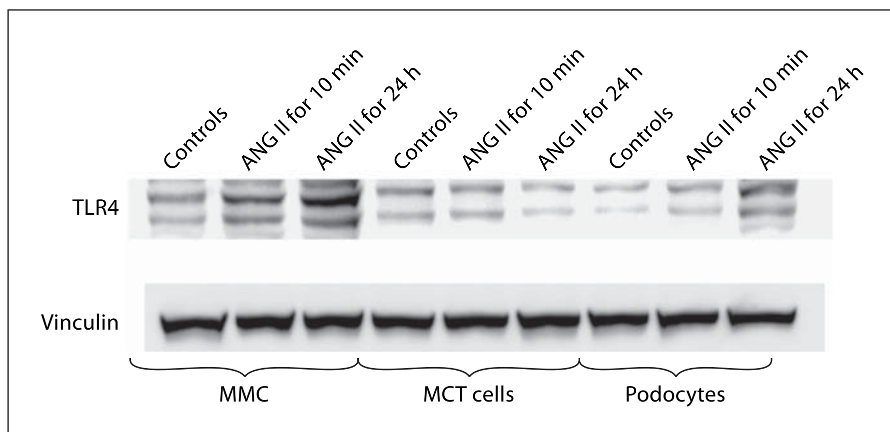

Fig. 2. Western blot analysis of the TLR4 expression in MMCs, podocytes, and MCT cells. ANG II (10^{-7} M for 24 h) stimulated the TLR4 protein expression in MMCs and podocytes, but not in MCT cells (upper panel). Equal loading was verified by analyzing the vinculin expression (lower panel). Results are representative of four independent experiments.

ANG II Increases the TLR4 Protein Expression in MMCs and Podocytes

Western blot analyses were performed to assess the effect of ANG II (10^{-7} M for 10 min and 24 h) on the TLR4 protein expression by MMCs, podocytes, and MCT cells. In accordance with results of other studies [9], Western blot analysis revealed two closely related bands of approximately 90 kDa, reflecting different degrees of TLR4 glycosylation (fig. 2). The basal TLR4 expression was similar in MMCs and MCT cells and slightly less in podocytes, but these changes did not reach statistical significance (MMC 0.85 ± 0.20, MCT cells 0.79 ± 0.31, podocytes 0.72 ± 0.29; absolute basal expression of TLR4 protein normalized to vinculin, n = 4, not significant). As shown in figure 2, ANG II differentially modulated the TLR4 protein expression in the three cell lines. In MMCs, ANG II induced a robust increase in the TLR4 protein expression (2.2 ± 0.3-fold and 1.7 ± 0.5-fold increase relative to controls 10 min and 24 h after ANG II stimulation, respectively, n = 4, $p < 0.01$ vs. controls for both). In podocytes, the ANG-II-mediated stimulation of the TLR4 protein expression was modest (1.2 ± 0.5- and 1.9 ± 0.61-fold increase relative to controls 10 min and 24 h after AG II stimulation, respectively, n = 4, $p < 0.05$ vs. controls after 24 h). Finally, ANG II did not significantly enhance the TLR4 expression in MCT cells (1.12 ± 0.23-fold and 1.05 ± 0.72-fold increase relative to controls 10 min and 24 h after ANG II stimulation, respectively, n = 4, $p > 0.05$ vs. controls).

ANG II Increases the TLR4 mRNA Expression in MMCs and Podocytes

Figure 3 shows that the TLR4 mRNA expression normalized to GAPDH as measured by real-time PCR in MMCs, podocytes, and MCT cells cultured for 24 h with or without 10^{-7} M ANG II. In agreement with Western blot analyses, ANG II significantly increased the TLR4 transcript levels in MMCs and podocytes, but not in MCT cells.

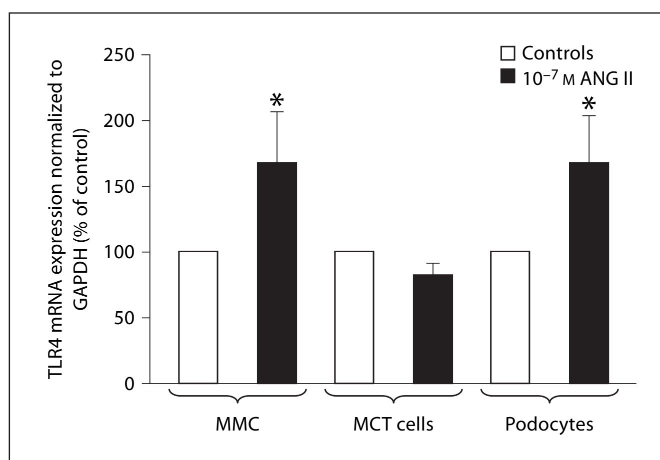

Fig. 3. Real-time PCR quantification of TLR4 mRNA expression in MMCs, podocytes, and MCT cells. ANG II (10^{-7} M for 24 h) significantly increased the TLR4 mRNA expression in MMCs and podocytes, but not in MCT cells. n = 9; * $p < 0.05$.

ANG II Increases the TLR4 Promoter Transcriptional Activity in MMCs and Podocytes, an Effect That Requires AP1 and PU.1 DNA-Binding Sites

To get insights into the mechanisms controlling the *Tlr4* gene regulation, we analyzed the transcriptional activity of wild-type and mutant TLR4 promoter luciferase reporter vectors in MMCs, podocytes, and MCT cells cultured either in medium or with ANG II (10^{-7} M for

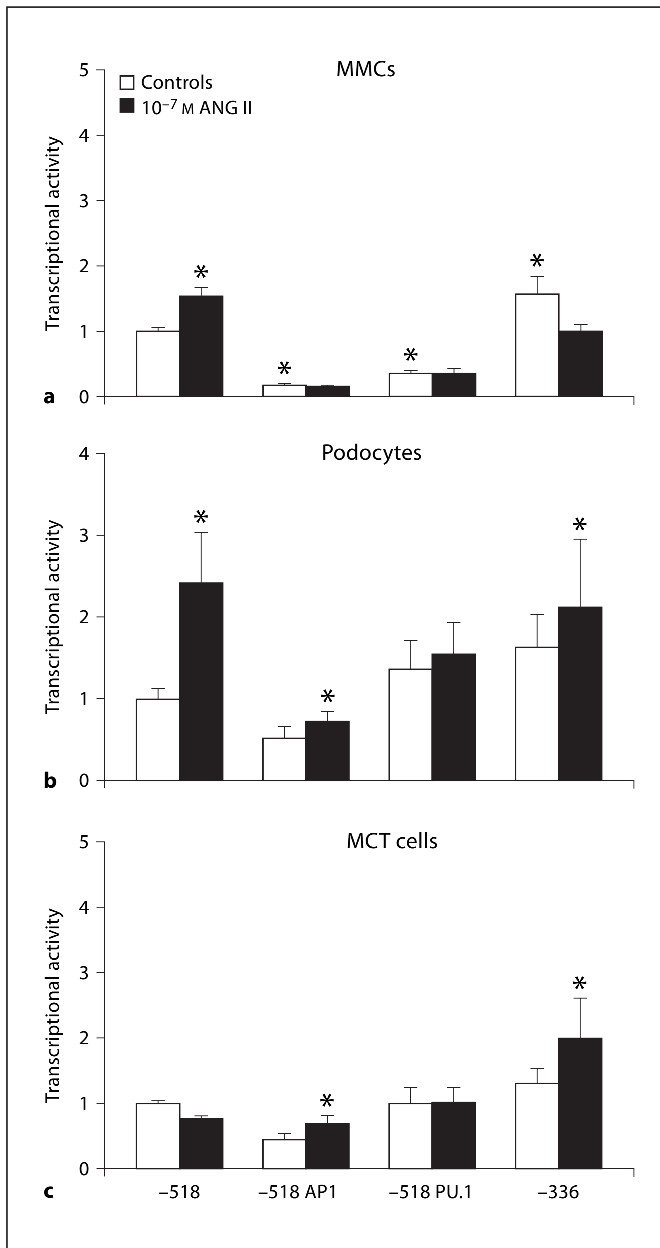

Fig. 4. TLR4 promoter transcriptional activity in MMCs (**a**), podocytes (**b**), and MCT cells (**c**). The basal transcriptional activity of the –518/+129 TLR4 promoter construct depended on AP1 and proximal PU.1 sites in MMCs and on AP1 in podocytes and MCT cells. ANG II (10^{-7} M for 24 h) significantly increased the luciferase activity of the –518/+129 construct in MMCs and podocytes, but not in MCT cells. This effect was abolished after mutation of the AP1 or the proximal PU.1 site. n = 4; * $p < 0.05$ versus –518/+129 controls without ANG II.

24 h). The wild-type promoter construct, ranging from –518 to +129 bp, was previously shown to contain all regulatory elements necessary for basal transcriptional activity in macrophages, fibroblasts, and endothelial cells [8]. The absolute expression of the –518/+129-bp promoter construct (luciferase normalized to β-galactosidase/40 μg protein, n = 15) under basal condition was significantly different in the three cell lines, with the highest expression in MMCs and the lowest in podocytes: MMCs 26.27 ± 6.80 ($p < 0.01$ vs. MCT cells and podocytes), MCT cells 18.81 ± 4.15 ($p < 0.05$ versus podocytes), and podocytes 10.46 ± 5.96. Figure 4 shows that in MMCs, podocytes, and MCT cells the basal transcriptional activity depended on an intact AP1 site. Additionally, the basal TLR4 promoter activity was enhanced by a proximal PU.1-binding site in MMCs. In agreement with RNA analyses (fig. 3), ANG II significantly stimulated the transcriptional activity of the –518/+129-bp promoter construct in MMCs and podocytes (fig. 4a, b), but not in MCT cells (fig. 4c). Considering that it has been previously shown that ANG II activates transcription factors that bind to AP1- and PU.1-binding sites [10, 11] and that the –518/+129-bp construct contains functional AP1 and PU.1 DNA-binding sites [8], these sites were mutated within the –518/+129-bp promoter. Disruption of either the AP1 site or the proximal PU.1 site abolished the ANG-II-mediated increased transcriptional activity in MMCs and podocytes (fig. 4).

Discussion

TLR4 has been found to reside in the kidney on mesangial and tubular cells, but its expression on podocytes was previously not described [2]. It has been previously shown that TLR4 plays an important role in renal damage under pathophysiological conditions such as sepsis and ischemic injury [10, 12, 13]. For example, Cunningham et al. [14] have shown by performing renal cross-transplantation between wild-type and TLR4 knockout mice that wild-type kidneys in $TLR4^{-/-}$ hosts are still susceptible to endotoxin-induced renal failure. These data suggest that intrarenal expression of TLR4 is important for the development of renal injury in this setting. Interestingly, extracellular matrix components, including fibronectin, hyaluronic acid, fibrinogen/fibrin, and fragments of heparan sulfate, can all activate TLR4 [for a review see ref. 13]. Consequently, in situations such as a rapidly progressive glomerulonephritis, destruction of the glomerular ultrafiltration barrier with the potential release of ex-

tracellular matrix components and mesangial deposition of fibrin may lead to activation of local upregulated TLR4 that mediates, in turn, glomerular inflammation. Although the pathophysiology of TLR4 within the kidney is only beginning to be understood, it is obvious that a better insight into the factors regulating the renal TLR4 expression is necessary.

We have previously observed that ANG II stimulates the expression of TLR4 on mesangial cells in vitro and in vivo and that this upregulation enhances lipopolysaccharide-induced nuclear factor kappa B activation and chemokine expression [3]. The present study was undertaken to investigate the regulation of the TLR4 expression by ANG II in three different mouse renal cell types and also to investigate the potential factors involved in transcriptional regulation. The major finding was that ANG II stimulates TLR4 mRNA and protein expression in MMC and podocytes, but not in MCT cells. This finding was not explained by differences in ANG II receptor subtypes, because all three cell types mainly expressed AT1b receptors and, to a lesser degree, AT1a, but not AT2 receptors [5, 6, 15]. The difference in ANG-II-mediated TLR4 expression in the three renal cell types is caused by differential transcriptional activation of the murine TLR4 promoter. The –518 construct of the mouse TLR4 gene 5′-flanking region contains putative binding sites for several transcription factors, including GATA, AP1, Oct, and three Ets sites [8]. ANG II has been previously found to activate the transcription factors AP1 and PU.1 in various cell types [9, 11]. ANG II activates the –518 construct only in MMC and podocytes, but not in MCT cells. The ANG-II-mediated transcriptional activation in MMC and podocytes is diminished in –518 constructs with mutated AP1- or PU.1-binding sites. This finding indicates that ANG-II-mediated activation of AP1- and PU.1-bindings factors is the major mediator of TLR4 transcriptional activation. Interestingly, basal transcription of TLR4 in the absence of ANG II in MMC and podocytes was also dependent on the presence of AP1- and PU.1-binding sites and was reduced after mutagenesis of these DNA-binding sites within the –518 construct. These findings are in accordance with observations demonstrating that corticotropin-releasing-factor-induced TLR4 expression in macrophages depends on the activation of PU.1 and AP-1 transcription factors [16].

The –336 construct does not contain the GATA site, and this region has been previously identified as a negative regulatory site, when the transcriptional activity of the –518 and –336 constructs was analyzed in various murine macrophage cell lines [8]. The GATA family of transcription factors is essential for cell maturation and differentiation within erythroid and megakaryocytic lineages [17]. Why this transcription factor should play a role in TLR4 regulation in nonhematological cells is currently unclear, but it seems that the negative effect of the GATA site plays also a role in renal cells, because the basal activity of the –336 construct was higher than that of the –518 construct, at least in MMCs and podocytes. However, ANG II failed to significantly increase the activation of the –336 construct in all three cell types. These observations suggest that additional sequences between –518 and –336 are necessary for the ANG-II-induced transcriptional activation of the *Tlr4* gene. Why ANG II fails to stimulate TLR4 transcription in MCT cells is currently unclear. We have previously shown that MMCs and MCT cells exhibit fundamentally different growth responses after challenge with ANG II. The peptide stimulates proliferation of MMCs, but mediates hypertrophy and cell cycle arrest in MCT cells [6, 9, 18]. Further studies are necessary to analyze differences in the ANG-II-induced transcription in MMCs/podocytes and MCT cells.

Intrarenal ANG II formation is stimulated in many acute and chronic renal diseases [18]. Accumulating evidence in the last years demonstrated that ANG II exhibits proinflammatory effects within the kidney and that ANG-converting enzyme inhibitors or AT1 receptor blockers have clearly anti-inflammatory properties [18, 19]. Part of the proinflammatory effects of ANG II in the kidney may be mediated by TLR4 expression, and a better understanding of the factors involved in ANG-II-mediated *Tlr4* gene transcription is essential for the development of innovative therapeutic strategies to interfere with chronic renal injury.

Acknowledgments

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