Candesartan attenuates Angiotensin II-induced mesangial cell apoptosis via TLR4/MyD88 pathway

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ABSTRACT

Angiotensin II (Ang II) can stimulate Toll-like receptor 4 (TLR4) expression in mesangial cells (MCs), but the role of TLR4 in the Ang II-induced apoptosis and the effect of candesartan on TLR4 expression remain unclear. Here, we report that Ang II-induced MC apoptosis in a time-dependent manner and up-regulated TLR4/MyD88 expression, and that the intracellular ROS was subsequently increased. We also show that candesartan attenuated the Ang II-induced MC apoptosis, and that this protective effect was dependent on decreased TLR4/MyD88 expression as well as reduced intracellular ROS formation. Furthermore, Ang II increased the apoptosis inducing factor protein level, while candesartan markedly reduced this increase. These results demonstrate that TLR4/MyD88 pathway was involved in the Ang II promoted MC apoptosis, which was related to TLR4/MyD88 mediated oxidative stress. These data also suggest that candesartan exerted anti-apoptotic effect as an antioxidant by modulating this pathway.

Angiotensin II (Ang II) plays a fundamental role in the progression of renal injury. In the renal glomerulus, mesangial cells (MC) are prone to Ang II-induced cell stress and lesion [1,2]. Several studies have suggested that Ang II causes mesangial injury by promoting cell loss (apoptosis), inflammation and extracellular matrix accumulation [2–4]. Among these pathologies, Ang II-induced reactive oxygen species (ROS) may play a pivotal role [1,2,4]. In most instances, apoptosis is an important process for normalizing the hypercellularity of injured glomeruli. However, under certain pathologic conditions, occurrence of excessive apoptosis may be an important event in the progression of glomerulosclerosis [5]. Therefore, Ang II-induced apoptosis of MC may facilitate the course of nephrosclerosis.

It has been demonstrated that increased Ang II activity is accountable for glomerular cell apoptosis [2]. This effect can not be explained only by alteration of hemodynamics [2,4], but the exact molecular mechanism remains to be clarified. Many detrimental effects of Ang II, including oxidative stress and apoptosis, have been attributed to the stimulation of the Ang II receptors (ATs) [2,4,5]. However, as we know, Ang II type 1 receptor (AT1R) blockers (ARBs) have the potential to reduce renal stress independent of their anti-hypertensive properties and the pharmacological mechanism remains to be under active consideration [6–8]. Particularly, candesartan (Cand), a highly selective ARB that can barely affect AT2R, has been reported to attenuate

oxidative stress in cells that lacking AT1R, which strongly suggests that the antioxidant capacity of Cand is independent of ATs [7].

Recently a number of studies have demonstrated the role of Toll-like receptors (TLRs) as pathogen-associated pattern recognition molecules in renal injury [9,10]. It has been shown that TLRs can facilitate harmful response through signaling cascade that involves myeloid differentiation factor 88 (MyD88), among others, which may lead to the activation of transcriptional factor NF-ĸB and subsequent regulation of immune and inflammatory genes [11]. TLR4 is the first identified molecule that mediates endotoxin-triggered signaling, and can recognize endogenous ligands such as heat shock proteins and extracellular matrix components in response to cellular damage [9,11]. Recent studies suggest that Ang II may serve as a "danger" factor by modifying TLR4 expression in MCs [13,14]. Moreover, TLR4 expression can be inhibited by candesartan both in vivo and in vitro [15,16]. On the other hand, it has been identified that ROS can be modulated by TLR4 and MyD88 signaling in several cell lines [17,18].

Since Ang II is a potent inducer of ROS in renal system[2,4], it is logical to speculate that Ang II may contribute to the MC injury through innate immune pathway based on the above findings. However, the role of TLR4 in the Ang II mediated MC loss and the direct effect of Cand on TLR4 expression in MCs have not been examined. Therefore, we analyzed the effects of high concentration of Ang II on the generation of ROS and early apoptosis in MCs, and elucidated the pathway that was involved in Ang II mediated MC injury. We further investigated whether candesartan can suppress

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early apoptosis in MCs and whether the suppression is via the TLR4/MyD88 pathway.

Materials and methods

Cell cultures. The rat mesangial cell line (HBZY-1) was purchased from China Center for Type Culture Collection (Wuhan, China) and grown in 25-cm² vent-cap tissue culture flasks containing DMEM supplemented with 10% fetal bovine serum (FBS) in 5% CO_2 at 37 °C. Cells were subcultured when the cell monolayer reached 80–90% confluence. MCs were rested for 12 h in DMEM with 1% FBS before adding Ang II with or without Cand or TLR4 blocking peptide (Santa Cruz, CA, USA).

Annexin V and propidium iodide staining. Apoptosis was determined by flow cytometry with annexin V-FITC and propidiumio-dide (PI) (BD Co. Ltd., USA). MCs were cultured for 48 h in 6 cm Petri dish (Corning, USA) and incubated in 1% FBS culture medium for 12 h. Cells were then exposed to 10^{-7} M Ang II for 0, 2, 4 and 8 h, or pre-incubated with 10^{-5} M Cand for 1 h and then treated with 10^{-7} M Ang II for 8 h. To determine the role of TLR4, equal numbers of MC were pre-incubated with 20 μg/ml TLR4 blocking peptide for 1 h and 10^{-7} M Ang II for 8 h, followed by apoptosis assay. Cells were trypsinized, centrifuged and washed twice with ice-cold PBS. Then the cells were resuspended in 400 μl cell suspension buffer and added into 5 ml centrifuge tube, followed by adding 5 μl of annexin V-FITC and 5 μl of PI. Cells were further incubated at room temperature for 15 min, and analyzed by EPICS ALTRAII flow cytometer (Beckman, USA) (excitation 488 nm). Ten thousand cells were collected for each sample. All experiments were repeated three times.

Detection of intracellular reactive oxygen species. Since high glucose could induce intracellular ROS [19], we used low glucose (5.5 mmol/l) and 1% FBS culture medium for cell synchronization and subsequent experiments. Coverslips of confluent cells were exposed to (a) Ang II (0 and 10^{-7} M) for 1 h, (b) 20 μg/ml TLR4 blocking peptide for 1 h followed by 10^{-7} M Ang II for 1 h, and (c) 10^{-5} M candesartan for 1 h followed by 10^{-7} M Ang II for 1 h. Intracellular ROS production was measured by the oxidation-sensitive DCFH-DA dye (Molecular Probes, USA), which was oxidized to the highly fluorescent 2′,7′-dichloro-fluorescein (DCF) by H_2O_2 or OH^- within the cell. Briefly, cells were incubated in the dark for 40 min at 37 °C after adding 10 μmol/l of DCFH-DA, and then washed three times with PBS. ROS generation was detected by a confocal microscope (Leica, Germany) (excitation, 488 nm; emission, 525 nm). All experiments were repeated three times.

Reverse transcription-polymerase chain reaction (RT-PCR). Cells were exposed to Ang II (10^{-7} M) for 15 min and 6 h, or pre-treated with 10^{-5} M candesartan for 1 h and then with 10^{-7} M Ang II for 15 min and 6 h, or untreated. RNA was extracted from the MCs using RNA-Solv reagent (Invitrogen, CA). Total RNA (2 μg) was used to synthesize the first-strand cDNA and served as a template for amplification of TLR4, MyD88 and GAPDH. The forward and reverse primer sequences (SBS Gene Tec, China) were as follows: TLR4, 5′-AAAGTTCTGACCGTTCTG and 5′-GTCCTCTCTGTTGGTAGTT; MyD88, 5′-AGCCGCCTCTCGCTGTTCTT and 5′-TGGGACACTGCTCTCCACTCT; GAPDH, 5′-ACGACCCCTTCATTGACCTCC and 5′-GCCAGTAGACTCCA CGACAT. The PCR products were 280, 327 and 200 bp, respectively. Amplification was carried out using MasterMix kit (Invitrogen, CA) in a total volume of 50 μl following the manufacturer's cycling parameters. PCR products were analyzed by electrophoresis on 1.8% agarose gel and the band intensities were determined using Image Quant Software.

Western blotting. Cells were treated as above for RT–PCR for indicated time points. When examined apoptosis inducing factor (AIF) activity, time point was 2 h. Proteins were extracted by 150 μl of RIPA lyse buffer (Beyotime, China), centrifuged at 12000g for 15 min at 4 °C, and the supernatant was collected. The protein content was measured using BCA protein assay kit (Pierce Bio, USA). The proteins were resolved under denaturing conditions in an 8% SDS–PAGE gel and electroblotted onto a nitro-cellulose membrane. The blotted membrane was incubated in 5% defatted milk in PBS with 0.1% Tween 20 for 1 h at 24 °C, and then incubated overnight at 4 °C with primary antibodies (Santa Cruz, CA). After washing three times, the membrane was incubated with horseradish peroxidase-conjugated anti-goat secondary antibodies (Santa Cruz, CA). The detection was performed using the ECL kit (Santa Cruz) according to the manufacturers' instructions. The intensity of the bands was analyzed with Alpha Ease FC image software. Each experiment was repeated three times.

Statistics. Statistical analysis was performed using SPSS software version 11.0 (Chicago, USA). All data are expressed as means ± SD. One-way ANOVA with appropriate post-hoc test was used to assess the statistical significance of differences. P value <0.05 was considered to be statistically significant.

Results

Induction of MC apoptosis by Ang II in a time-dependant manner

After Annexin V and PI double staining, induction of MC apoptosis by Ang II was confirmed by flow cytometry and was shown in a time-dependant manner. As shown in Fig. 1, the longer the time of MCs treated by Ang II, the higher the apoptosis rate was. Percentage of cells in late stage of apoptosis was much lower than that in early stage, indicating early apoptosis was predominant in MCs stimulated by Ang II during the 8 h treatment. When MCs were pre-treated with 10^{-5} M of Cand and then with Ang II, the rate of both early and late apoptosis decreased dramatically compared with that of Ang II treatment alone at 8 h ($p < 0.01$). Similarly, TLR4 blocking peptide pre-treatment followed by Ang II treatment also attenuated MC apoptosis.

Effect of Ang II with/without candesartan or blocking peptide on the production of ROS in MCs

As shown in Fig. 2, the exposure of MC to Ang II for 1 h was associated with a significant increase of intracellular ROS generation ($p < 0.01$ versus control). Meanwhile, ROS production was decreased significantly in Cand and TLR4 blocking peptide pre-treated groups (both $p < 0.01$ vs. Ang II treatment alone).

Effect of Ang II with/without candesartan on TLR4 and MyD88 mRNA expression in MCs

The effect of Ang II on the mRNA expression of TLR4 and MyD88 was analyzed by RT-PCR. As shown in Fig. 3, untreated MC expressed low levels of TLR4 and MyD88 mRNA, whereas 10^{-7} M Ang II treatment up-regulated TLR4 and MyD88 mRNA rapidly and persistently. Ang II up-regulated TLR4 mRNA expression by 1.8-fold and 2.1-fold at 15 min and 6 h, respectively, while MyD88 mRNA expression increased 1.7-fold and 2.3-fold at the same time points. On the other hand, Cand significantly reduced Ang II up-regulated TLR4 and MyD88 mRNA expression (both $p < 0.01$ vs. Ang II treatment alone).

Upregulation of TLR4 and AIF proteins by Ang II in MCs and the abrogation of the upshift by candesartan

The effects of Ang II on TLR4 and AIF proteins were analyzed by Western blot. As shown in Fig. 4A, TLR4 protein was increased 1.7- and 2.2-fold by Ang II at 15 min and 6 h, respectively. Pre-treat-
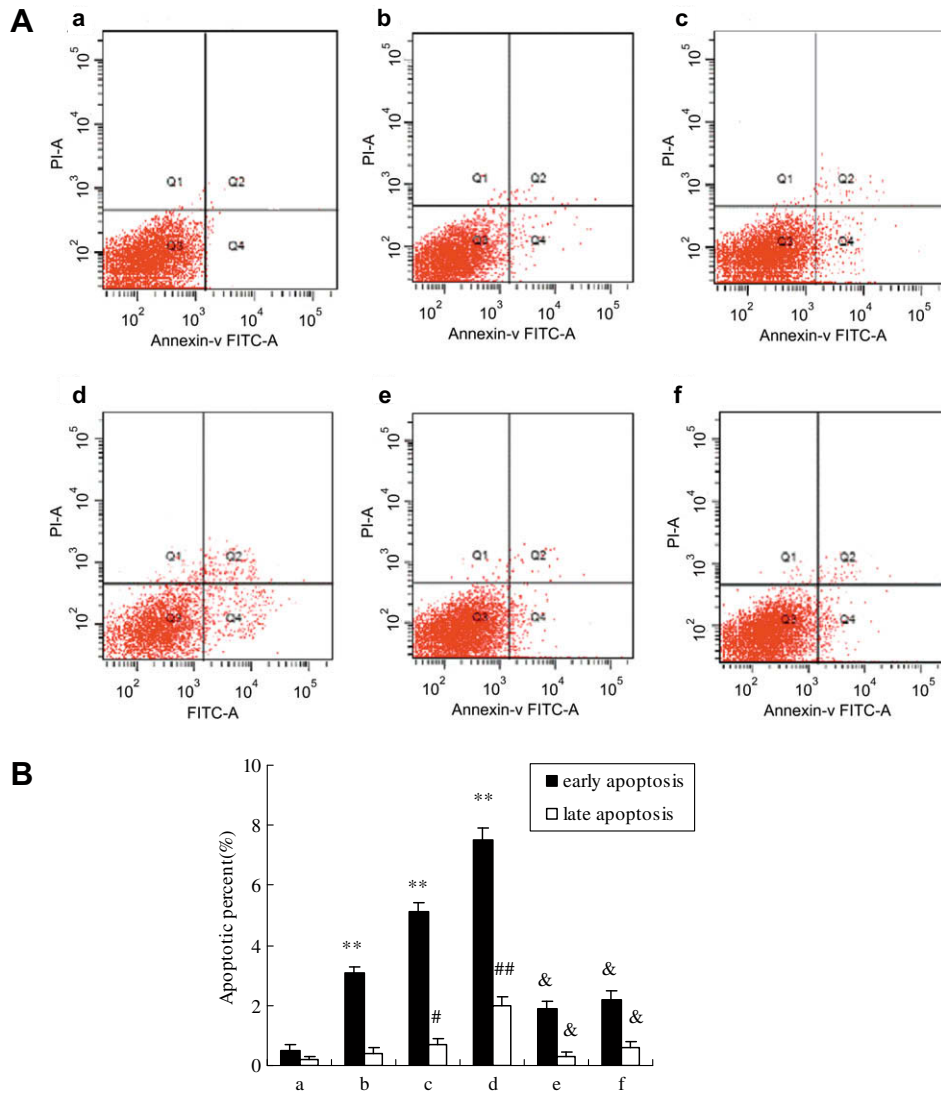
Fig. 1. Induction of apoptosis by Angiotensin II in MCs. (A) Flow cytometry chart after Annexin V and PI double staining. a, untreated cells; b–d, cells exposed to 10^{-7} M Ang II for 2, 4 and 8 h, respectively; e–f, cells pre-treated with 10^{-5} M Cand or 20 µg/ml TLR4 blocking peptide for 1 h and then with 10^{-7} M Ang II for 8 h, respectively. (B) Apoptotic rate of the above treatments. Data show the means ± SD ($n = 4$); a–f, as above. $^{**}p < 0.01$ compared to early apoptotic cells in control group. $^{#}p < 0.05$ and $^{##}p < 0.01$ compared to late apoptotic cells in control group. $^{&}p < 0.01$ compared to Ang II treatment 8 h.

ment with 10^{-5} M Cand markedly attenuated TLR4 protein level ($p < 0.05$ in 15 min and $p < 0.01$ in 6 h vs. Ang II treatment alone, respectively), Cand also abrogated the upshift of AIF protein enhanced by Ang II (Fig. 4B).

Discussion

Although various detrimental effects of Ang II, including MC loss, have been suggested, the molecular details are not completely understood. The present study demonstrated that Ang II may induce MC apoptosis in a time-dependent manner irrespective of its hemodynamic effects. Because Ang II has the potential to upregulate ROS production in MCs, it enables ROS to act as an important contributor of Ang II-induced MC apoptosis [2,4]. Based on the evidences that Cand suppressed renal oxidative stress independent of AT$_1$R blockade [7] and that Cand could inhibit TLR4 expression in vitro [15,16], it is likely that the Ang II-induced renal cell injury also involves an AT receptor independent mechanism. Since TLR4 can be activated in dangerous conditions even in the absence of infection [10,12,13], we speculate that TLR4 may play an important

role in the Ang II-induced pathophysiologic process in the kidney. In agreement with a recent report of Bondeva et al. [13], our study showed that Ang II evoked a rampant and persistent TLR4 expression in MCs (Figs. 3 and 4A). Furthermore, we found that MyD88, the proximal adaptor molecule of TLR4, was also upshifted within a similar time span of TLR4 (Fig. 3), and these upregulations may result in oxidative stress and apoptosis. Strikingly, candesartan alleviated Ang II-induced TLR4/MyD88 expression significantly, while TLR4 blockade partially attenuated ROS and apoptosis. These findings suggest that TLR4/MyD88 pathway may be involved in the process of Ang II-induced MC impairment, and that Cand may have direct protective effects on MCs.

Candesartan, a highly selective ARB, has been extensively studied for its beneficial effects on renal and cardiovascular injury. However, in most studies, the protective effects of Cand were attributed to blockade of AT1R, whereas our data suggest that TLR4 was implicated in Ang II-induced stress in early stage of Ang II stimulation, Cand may help normalize ROS production by modulation of TLR4/MyD88 pathway in MCs and yield an antioxidant-like effect (Fig. 2). In vivo studies have also suggested that the
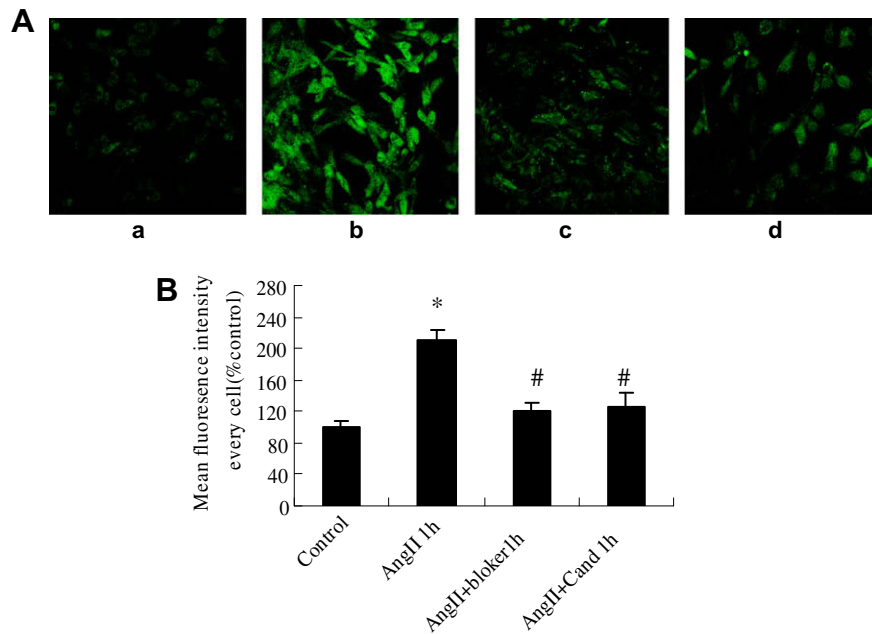
Fig. 2. Effects of Angiotensin II with/without candesartan or TLR4 blocking peptide on ROS production in MCs. (A) ROS images obtained by confocal microscopy. a, untreated cells; b, cells treated with 10^{-7} M Ang II for 1 h; c–d, cells pre-treated with 20 µg/ml TLR4 blocking peptide or 10^{-5} M Cand for 1 h and then with 10^{-7} M Ang II for 1 h, respectively. (B) ROS production ($n = 12$). $^*p < 0.01$ versus control, $^\#p < 0.01$ versus Ang II group.
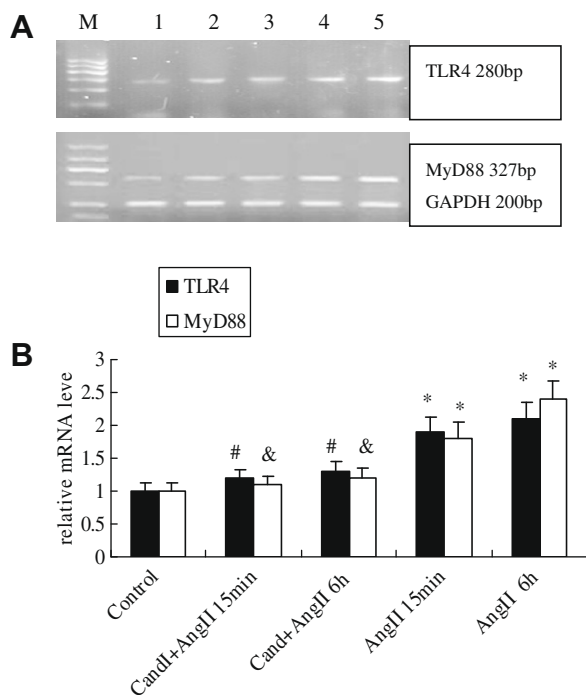

Fig. 3. Induction of TLR4 and MyD88 mRNA with candesartan and/or Angiotensin II in MCs. (A) RT-PCR results. 1, control; 2–3, Cand pre-incubated + Ang II treatment for 15 min, 6 h, respectively; 4–5, Ang II treatment for 15 min, 6 h, respectively. (B) Relative mRNA level of TLR4 and MyD88, which were normalized with GAPDH levels. $^*p < 0.01$ versus control; $^\#p < 0.01$ versus Ang II treatment for 15 min and 6 h (TLR4), respectively; $^\&p < 0.01$ versus Ang II treatment for 15 min and 6 h (MyD88), respectively.

antioxidant effect of ARBs may not depend on AT1R because Cand is able to modulate accumulation of ROS and improve endothelial function in patients without hypertension [23,24]. Consistent with these clinical studies, our in vitro study showed that Cand attenuated ROS and subsequent apoptosis in MCs induced by Ang II (Figs.

1 and 2). These results indicate that Cand has antioxidant effect, which may account for its prevention against MC apoptosis elicited by Ang II.

The role of TLRs in the onset and development of renal diseases has been increasingly recognized [9–12]. In the present study, we found that Ang II significantly increased cellular ROS in MCs (Fig. 2), and that induction of TLR4/MyD88 expression preceded the release of ROS by Ang II (data not shown). These observations suggest that TLR4/MyD88 pathway may contribute to ROS generation in response to Ang II. This is supported by other studies using different types of cells, which demonstrate that TLR4 and its upstream components, including MyD88, are involved in the oxidative stress [17,18]. Evidences linking TLR4 and ROS also come from observations documenting that NADPH-oxidase (Nox) can be modulated by Ang II-enhanced TLR4 signaling through transcription factors such as Ets [20–22].

ROS has been identified to be linked to the activation of apoptosis in several cell lines including MC [2,25]. For example, Lodha et al. have demonstrated that oxidative stress plays a causal role in Ang II-induced MC apoptosis, and antioxidants could ameliorate this response [2]. Matsuzawa et al confirmed that TLR4 mediated ROS act as transducers of apoptosis [26]. Together with the current study, these observations suggest that Ang II-induced MC apoptosis might be due to the activation of mitochondrial "intrinsic" pathways, which is usually triggered by increased free radical production and followed by release of proapoptotic proteins such as AIF and cytochrome *c* from the mitochondria into the cytosol [25,27,28]. Of note, in response to ROS stimuli and precedent to cytochrome *c* release, AIF is released from mitochondria into the cytosol during apoptosis, translocated to the nucleus, and participated in the induction of chromatin condensation and the exposure of phosphatidylserine (PS) in the outer leaf of the plasma membrane [27,29]. In our study, we examined the early apoptotic event by Annexin V staining. Since Annexin V is a phospholipid binding protein that has a high affinity for PS and externalization of PS is a specific event in the early stage of apoptosis, Annexin V staining can identify apoptotic cells early. We found that apoptosis started as early as 2 h after Ang II treatment and promoted gradu-
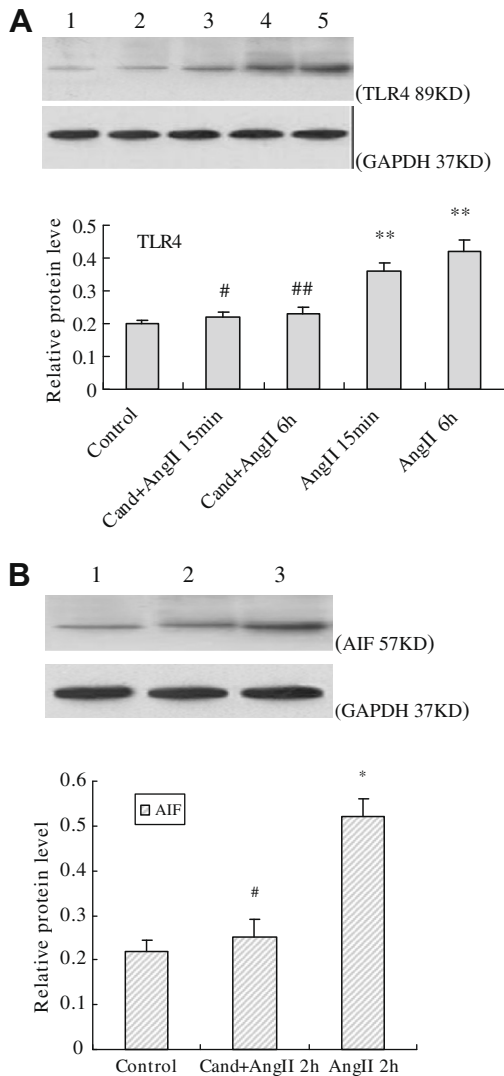
A

B

Fig. 4. Effects of candesartan and/or Angiotensin II on TLR4 and AIF protein level in MCs. (A) Western-blot of TLR4 protein. Relative protein level of TLR4 was normalized to GAPDH. $^{**}p < 0.01$ versus control. $^{#}p < 0.05$ versus Ang II treatment for 15 min, $^{##}p < 0.01$ versus Ang II treatment for 6 h, respectively. (B) Western-blot of AIF protein. Relative protein level of AIF was normalized to GAPDH. $^{*}p < 0.01$ versus control, $^{#}p < 0.01$ versus Ang II treatment for 2 h.

ally (Fig. 1), we also observed chromatin condensation in Ang II-treated MCs by hoechst staining (data not shown). These early events were coincident with the upshift of AIF, indicating that AIF played an important accelerating role in early apoptosis. Further, TLR4 blockade partially attenuated ROS formation and apoptosis (Figs. 1 and 2), and therefore TLR4 may have a critical role in the oxidative stress-associated apoptosis.

A previous report has suggested that apoptosis occurs after 18–24 h Ang II treatment in MCs and might be modulated by ATs [2]. Therefore, it is possible that Ang II-induced apoptosis may have two stages: an early apoptosis that is mediated by the innate immune pathway, and a late apoptosis that involves AT receptors. This is supported by the finding that 10^{-7} M Ang II did not change the AT1 and AT2 mRNA expression in MCs within 24 h [13]. Although we could not rule out the possibility of TLR4 to cross-talk with other receptors, the current study clearly demonstrate that TLR4/MyD88 pathway was involved in Ang II-induced MC oxidative stress and early apoptosis, and that Cand suppressed ROS formation by modulating this pathway and thus preventing MCs from Ang II-induced apoptosis.

In conclusion, we provided evidence that TLR4/MyD88 was an essential pathway that promoted MC oxidative injury and the subsequent apoptosis in response to Ang II stimulation, and that candesartan exerted protective effects as an antioxidant via this pathway. To our knowledge, this is the first report revealing the critical role of innate immune molecules in Ang II-induced renal cell apoptosis and the novel pharmacological mechanism of candesartan. These results may have implications in the treatment of nephrosclerosis.

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