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Macrophage-derived IL-6 contributes to ANG II-mediated angiotensinogen stimulation in renal proximal tubular cells

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O’Leary R, Penrose H, Miyata K, Satou R. Macrophage-derived IL-6 contributes to ANG II-mediated angiotensinogen stimulation in renal proximal tubular cells. Am J Physiol Renal Physiol 310: F1000–F1007, 2016. First published March 23, 2016; doi:10.1152/ajprenal.00482.2015.—The development of ANG II-dependent hypertension involves increased infiltration of macrophages (MΦ) and T cells into the kidney and the consequent elevation of intrarenal cytokines including IL-6, which facilitates the progression of hypertension and associated kidney injury. Intrarenal renin-angiotensin system (RAS) activation, including proximal tubular angiotensinogen (AGT) stimulation, has also been regarded as a cardinal mechanism contributing to these diseases. However, the interaction between immune cells and intrarenal RAS activation has not been fully delineated. Therefore, the present study investigated whether ANG II-treated MΦ induce AGT upregulation in renal proximal tubular cells (PTCs). MΦ were treated with 0–10^−6 M ANG II for up to 48 h. PTCs were incubated with the collected medium from MΦ. In ANG II-treated MΦ, IL-6 mRNA and protein levels were increased (1.85 ± 0.14, protein level, ratio to control); moreover, IL-6 levels were higher than TNF-α and IL-1β in culture medium isolated from ANG II-treated MΦ. Elevated AGT expression (1.69 ± 0.04, ratio to control) accompanied by phosphorylated STAT3 were observed in PTCs that received culture medium from ANG II-treated MΦ. The addition of a neutralizing IL-6 antibody to the collected medium attenuated phosphorylation of STAT3 and AGT augmentation in PTCs. Furthermore, a Jak2 inhibitor also suppressed STAT3 phosphorylation and AGT augmentation in PTCs.

These results demonstrate that ANG II-induced IL-6 elevation in MΦ enhances activation of the JAK-STAT pathway and consequent AGT upregulation in PTCs, suggesting involvement of an immune response in driving intrarenal RAS activity.

interleukin-6; angiotensin II; angiotensinogen; macrophage; renin-angiotensin system

HYPERTENSION accounts for approximately one-fourth of all heart failure, with 60% of these cases attributable to hypertension in the elderly (46). Since the intrarenal renin-angiotensin system (RAS) plays cardinal roles in controlling blood pressure and regulating electrolyte and body fluid homeostasis (27, 28), an activated RAS in the kidney is a major risk factor contributing to the development of hypertension and kidney injury. A key process contributing to the elevation of intrarenal ANG II is augmentation of intrarenal angiotensinogen (AGT) (28), which is primarily produced in renal proximal tubular cells (PTCs) (11, 16, 41). In particular, renal proximal tubule-specific overexpression of AGT amplifies intrarenal ANG II levels and causes the development of hypertension and kidney injury (36, 48). Thus, elucidating the mechanisms that regulate intrarenal AGT is essential for the development of novel strategies to prevent and treat hypertension and RAS-associated tissue injury.

ANG II promotes AGT augmentation in the kidney as well as in the heart, liver, and adipose tissue (3, 7, 14, 21, 23, 43). Thus, the intrarenal amplification of AGT by ANG II has been regarded as a pivotal mechanism facilitating the progression of hypertension. However, some in vitro studies using cultured human PTCs have failed to show AGT upregulation by direct treatment with ANG II (37, 38), suggesting that a mediator is required for the intrarenal AGT augmentation observed in ANG II-dependent hypertension (7, 16).

Chronic ANG II elevation induces increased immune cell infiltration in the kidneys, contributing to increases in intrarenal cytokine levels. In particular, IL-6 is produced in high quantities by activated macrophages (MΦ) driving inflammatory processes (8, 12, 15). Previous studies have shown that both plasma and intrarenal IL-6 levels are markedly elevated in chronic ANG II-infused animals (34, 35, 51), and, in IL-6 knockout (KO) mice, the severity of ANG II-induced hypertension and activation of STAT3 activity are reduced (2, 20). Moreover, IL-6 KO improves the development of albuminuria and renal fibrosis in ANG II-induced hypertension (24, 52). These results suggest that IL-6 and its signaling pathway are involved in the development of ANG II-induced hypertension and kidney injury.

IL-6 stimulates the secretion (30) and expression of hepatic AGT by activating the JAK-STAT pathway (13, 33). Notably, IL-6 and STAT3 activation are also required for ANG II-induced AGT augmentation in PTCs (37). Together, these findings provide a firm basis for our hypothesis that elevated IL-6 production by activated immune cells, especially MΦ, leads to intrarenal AGT augmentation via activation of the JAK-STAT pathway ultimately facilitating intrarenal RAS activation during the development of ANG II-dependent hypertension. However, in ANG II-dependent hypertensive animal models, ANG II is responsible for stimulating the production of multiple pathogenic factors, including cytokines, hormones, oxidative stress, mechanical stress, and immune cells other than MΦ. Moreover, immunosuppression broadly and nonselectively alters immune cell compositions and the consequent pathophysiological status, causing difficulty in distinguishing the exact nature of the roles that activated MΦ and IL-6 play in intrarenal AGT regulation. Therefore, in an in vitro setting using cultured MΦ and PTCs, we aimed to
determine the role of IL-6 in proximal tubular AGT augmentation that occurs in ANG II-dependent hypertension.

MATERIALS AND METHODS

Cell culture. Commercially available rat MΦ (male, NR8383, American Type Culture Collection) and immortalized rat PTCs (male) (10) were used in this study. Since immortalized PTCs proliferated over monolayer levels during tested treatments of MΦ (up to 48 h), coculture systems by transwell plates were not used, and, therefore, a sequential culture system was used in the present study. MΦ were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated FCS (Invitrogen). MΦ were plated at a density of 1 × 10⁶ cells/well in 12-well plates. Before stimulation, cells were serum starved for 24 h. MΦ were treated with 0–10⁻⁶ M ANG II (Phoenix Pharmaceuticals, Phoenix, AZ) for up to 48 h. Thereafter, culture media from MΦ were collected and filtered using 0.22-μm filters to remove floating MΦ from the collected media. PTCs were subsequently treated with the collected medium for 24 h. MΦ that were not treated with ANG II (0 μM ANG II) and PTCs that did not receive culture medium from MΦ served as control groups. To test the direct effects of ANG II on AGT regulation in PTCs, PTCs were incubated with 100 nM ANG II for 24 h.

Cytokine ELISA. Cytokine levels of macrophage cell media were analyzed via sandwich ELISA using a commercially available kit (Signosis, Santa Clara, CA) after the manufacturer’s instructions. Briefly, 100 μl of standard, control, or sample were added to the proper well, incubated for 1 h, and washed before the addition of 100 μl of biotin-labeled antibody. After 1 h, excess antibody was removed via a wash. Wells were then probed with 100 μl of a streptavidin-horseradish peroxidase conjugate for 45 min before being washed. Substrate (100 μl) was added to begin the colorimetric reaction. The reaction was allowed to proceed for <30 min before the addition of stop solution and reading of the optical density at 450 nm.

Quantitative real-time RT-PCR. Quantitative real-time RT-PCR was performed to evaluate rat IL-6 and AGT mRNA levels using the TaqMan PCR system. For total RNA isolation, treated cells were washed with 3 ml PBS. PBS was aspirated, and total RNA was isolated from cells using the RNeasy kit (Qiagen). Subsequently, quantitative real-time RT-PCR was performed as previously described (17, 38). All samples were analyzed in triplicate, and the data were normalized based on expression levels of rat β-actin mRNA.

Western blot analysis. IL-6 and AGT protein levels were determined by Western blot analyses. Phosphorylation levels of STAT3 and expression levels of STAT3 were also detected by Western blot.
analyses to elucidate participation of these transcription factors in AGT regulation. The Western blots were performed as previously described (40). After treatment, cells were harvested with 80 μl lysis buffer containing 1% Triton X-100, 150 mmol/l NaCl, 1 mmol/l EDTA, 1% Nonidet P-40, 1 mmol/l Na3VO4, and 0.25% protease inhibitor cocktail (Sigma, St. Louis, MO). Lysates were sonicated three times for 10 s each and centrifuged at 13,000 rpm at 4°C for 30 min. Total protein concentration of the supernatant was quantified using a Micro BCA Protein Assay Kit (Pierce, Rockford, IL). Then, 20 μg of total protein were applied to a precast NuPAGE 4–12% gel (Invitrogen). The separated proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). After incubation of the membrane with primary and secondary antibodies, detection and analysis of protein were performed using the Odyssey System (Life Technologies, Frederick, MD). The separated proteins were transferred to a nitrocellulose membrane with primary and secondary antibodies, detection and analysis of protein were performed using the Odyssey System (Life Technologies, Frederick, MD). The separated proteins were transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). After incubation of the membrane with primary and secondary antibodies, detection and analysis of protein were performed using the Odyssey System (Life Technologies, Frederick, MD).

**Immunofluorescence staining.** PTCs were cultured in four-well chambers (Lab-Tek). After treatment with culture medium of MΦ (CMM) or ANG II-treated CMM (ANG II-CMM), cells were rinsed with PBS and then fixed for 20 min with 4% paraformaldehyde. After 4 min of incubation with 0.2% Triton X-100, the blocking agent Image-iT FX signal enhancer (Invitrogen) was added to the chambers. Cells were incubated with an antibody against STAT3 for 3 h. After being washed with PBS, cells were incubated with an Alexa fluor 594-labeled secondary antibody. ProLong Gold antifade reagent with 4',6-diamidino-2-phenylindole (Invitrogen) was used as a nuclear stain and a mount reagent. Localization of the stained proteins was observed and photographed under a fluorescence microscope (Olympus BX51, Olympus Optical).

**Transfection of suppressor of cytokine signaling 3.** Rat suppressor of cytokine signaling (SOCS3) cDNA was cloned by standard RT-PCR from rat kidney RNA isolates. The sense primer (5'-CTCCGT-GGCCCATGTCGTA-3') and antisense primer (5'-GAGCATCAT-ACTGGTCAGAAGACTC-3') were designed based on the rat SOCS3 complete coding sequence (National Center for Biotechnology Information Information no. AF075383.1) and used in PCR. The amplified SOCS3 cDNA by PCR was subcloned into the mammalian expression vector pcDNA3.1/CT-GFP-TOPO (Invitrogen) in both sense and antisense orientations. pcDNA3.1/CT-GFP-TOPO plasmids containing SOCS3 cDNA in sense and antisense orientations were transfected into PTCs with Lipofectamine reagents.

**Statistical analysis.** Data are expressed as means ± SE. Data were analyzed using a Student’s t-test or one-way ANOVA followed by a post hoc Bonferroni/Dunn multiple-comparison test. P values of <0.05 were considered statistically significant.

**RESULTS**

**Regulation of extracellular IL-6, TNF-α, and IL-1β levels by ANG II in MΦ.** The effect of ANG II on changes in extracellular IL-6, TNF-α, and IL-1β levels, which can serve as pathogenic factors in RAS-associated kidney injury (29, 35, 50, 52), in MΦ was investigated using a cytokine ELISA. In culture media from untreated MΦ, IL-6 was present in greater amounts than TNF-α and IL-1β: 0.52 ± 0.07 ng/ml, TNF-α: 0.14 ± 0.004 ng/ml, and IL-1β: 0.20 ± 0.03 ng/ml, n = 4; Fig. 1). IL-6 levels were elevated after 1 μM ANG II

![Fig. 3. Involvement of ANG II type 1 receptors (AT1Rs) in ANG II-induced IL-6 augmentation in MΦ. MΦ were treated with 1 μM ANG II for 48 h. After the treatment, AT1R mRNA (A) and protein (B) levels were determined by quantitative real-time RT-PCR and Western blot analysis, respectively. Furthermore, MΦ were pretreated with olmesartan (C) to test if AT1Rs mediate ANG II-induced IL-6 upregulation. Data are expressed as means ± SE. *Significant difference compared with each control group (P < 0.05); #significant difference compared with the ANG II-treated group (P < 0.05).](http://ajprenal.physiology.org/)
treatment for 48 h (0.97 ± 0.08 ng/ml). Although TNF-α and IL-1β levels in CMM were also increased by ANG II, IL-6 levels were most abundant among the tested cytokines.

Augmentation of intracellular IL-6 levels in ANG II-treated MΦ. Further analyses for IL-6 regulation by ANG II in MΦ were performed using several concentrations of ANG II at early (6 h) and late (48 h) time points. After 48 h of treatment, IL-6 mRNA levels and intracellular protein levels were augmented by 10⁻⁸ M (mRNA: 1.67 ± 0.11-fold and protein: 2.05 ± 0.09-fold, ratio to each control) and 10⁻⁶ M (mRNA: 2.18 ± 0.24-fold and protein: 3.43 ± 0.20-fold, ratio to each control) ANG II (n = 4; Fig. 2, A and B) compared with untreated (0 μM ANG II) MΦ. A lower concentration of ANG II (10⁻¹⁰ M) induced minor increases in IL-6 mRNA and intracellular protein upregulation, but they were not statistically significant. Whereas elevation of IL-6 mRNA was observed in ANG II-treated MΦ at 48 h, no change occurred after 6 h of treatment (Fig. 2C). Therefore, treatment of MΦ with ANG II for 48 h was used throughout the remainder of our study.

Involvement of ANG II type 1 receptors in ANG II-induced IL-6 augmentation in MΦ. First, we tested if ANG II alters ANG II type 1 receptor (AT₁R) expression levels in MΦ. Since specificities of commercial anti-AT₁R antibodies are controversial (9), AT₁R mRNA levels were measured by quantitative real-time RT-PCR and protein levels by Western blot analysis. No changes in AT₁R levels after ANG II treatment were observed in these analyses (n = 4; Fig. 3, A and B). MΦ pretreated for 1 h with 1 μM olmesartan (Sankyo) (37), an AT₁R blocker, showed significant attenuation of IL-6 augmentation after ANG II treatment (n = 4; Fig. 3C), suggesting that AT₁Rs mediate ANG II-induced IL-6 upregulation in MΦ.

Stimulating effects of CMM on AGT expression in PTCs. In PTCs, AGT mRNA expression levels were 1.41 ± 0.07-fold increased by CMM (n = 4; Fig. 4A) compared with AGT mRNA levels in untreated PTCs (control). Augmentation of AGT mRNA expression was further enhanced when PTCs received ANG II-CMM (2.39 ± 0.05-fold, ratio to control). In contrast, direct treatment of PTCs with 100 nM ANG II did not alter AGT mRNA (0.95 ± 0.13, ratio to control, n = 6) or protein (0.96 ± 0.11, ratio to control, n = 6) levels under the experimental conditions. An IL-6-neutralizing antibody (R&D Systems) added to collected ANG II-CMM significantly suppressed the elevation of AGT mRNA expression, indicating that IL-6 mediates AGT augmentation in PTCs treated with ANG II-CMM. However, AGT expression in PTCs treated with the anti-IL-6 antibody remained slightly higher than control levels (Fig. 4A) even when a higher concentration of the antibody was used. Regulation of AGT protein levels corresponded closely to changes in mRNA levels (Fig. 4B).

Activation of STAT3 by ANG II-CMM in PTCs. Since IL-6 has been characterized as a strong stimulator of the JAK-STAT pathway, STAT3 activity was evaluated in PTCs. CMM slightly increased phosphorylation levels of STAT3 in PTC (1.43 ± 0.09-fold, ratio to control, n = 4; Fig. 5A). ANG II-CMM treatment induced further phosphorylation in PTCs (2.76 ± 0.23-fold), which was prevented by the IL-6-neutralizing antibody. Immunocytochemistry demonstrated translocation of STAT3 into nuclei after incubation with ANG II-CMM (Fig. 5B).

Implications of the activated JAK-STAT pathway in AGT augmentation. STAT3 phosphorylation and AGT expression were facilitated by CMM and ANG II-CMM (n = 4 in each experiment; Fig. 6, A and B) as shown above. Pretreatment of PTCs with 1 μM AG-490, a JAK2 inhibitor, diminished STAT3 phosphorylation induced by both CMM and ANG II-CMM (Fig. 6A) to control levels (or slightly lower than the control). AGT augmentation by CMM and ANG II-CMM was also attenuated to the control levels (Fig. 6B), although suppression of AGT levels by an IL-6-neutralizing antibody was still greater than the control (Fig. 4). Overexpression of SOCS3-GFP (Fig. 6C), an endogenous suppressor of the JAK-STAT pathway, prevented AGT upregulation in PTCs treated by 10.220.32.247 on August 15, 2017 http://ajprenal.physiology.org/ Downloaded from
with CMM and ANG II-CMM, reinforcing JAK-STAT signaling as a critical mediator in AGT expression.

DISCUSSION

Since intrarenal ANG II is elevated in many forms of hypertension, the renal RAS is acknowledged as a key target for clinical and biochemical studies (27). Importantly, animal studies have demonstrated that elevated ANG II stimulates renal cortical AGT production, amplifying intrarenal RAS activity during the development of ANG II-dependent hypertension (7, 16). Recent studies have reported that chronic ANG II infusion in mice concomitantly increases AGT and IL-6 levels in renal cortical tubules (28, 35) and that IL-6 is required to augment AGT expression in cultured PTCs (37). The elevation of IL-6 levels in ANG II-dependent hypertension and stimulating effects of IL-6 on AGT expression have been demonstrated in other tissues (6, 13, 33, 34), suggesting the importance of elevated IL-6 in local RAS activation and the development of hypertension. This view is further supported by previous studies that revealed gene deletion of IL-6 in mice abrogates ANG II-induced hypertension (2, 20). During the development of hypertension mediated by elevated ANG II, infiltration of macrophages, which are a primary source of IL-6, and T cells is enhanced in the kidney (32, 53) as well as the vascular endothelium (6). These macrophages and T cells have been shown to play crucial roles in accelerating the progression of hypertension and associated tissue damages including kidney injury (25, 42, 44, 45, 49).

Previously, conflicting studies describing the role that ANG II plays in IL-6 expression in macrophages have been presented. In one study (8), it was shown that 1 μM ANG II enhances IL-6 production at 6 h in mouse macrophages, whereas other work (22) using the same dose of ANG II demonstrated an inhibitory effect of lipopolysaccharide-induced IL-6 augmentation in mouse macrophages. Thus, the activating effect of ANG II on IL-6 expression in macrophages was controversial. In the present study, ANG II increased IL-6 mRNA and protein levels in cultured rat macrophages as well as levels of the cytokine secreted in CMM. While TNF-α and IL-1β concentrations were increased in ANG II-treated CMM, IL-6 levels were significantly elevated, suggesting that IL-6 is one of the core proinflammatory cytokines in AGT regulation produced by ANG II-activated macrophages. In the present study, basal levels of IL-6 in the control group were elevated at 48 h compared with 6 h. The augmentation could be due to a number of things, including autocrine and paracrine signaling induced by secreted factors from these macrophages during the 48-h incubation. Although there is no doubt that TNF-α and IL-1β contribute to the development of kidney injury, including renal inflammation, these cytokines have been reported to suppress AGT in PTCs (38, 40). Accordingly, our present study highlights the significant impact of IL-6 on AGT upregulation. In these experiments, a high dose of ANG II (>10 nM) and a long-term treatment duration were required to induce significant IL-6 augmentation in macrophages, findings in agreement with a previous study (8). This effect might be due to the use of only a single treatment of cells at the beginning of the experiment.

Fig. 5. Activation of STAT3 by ANG II-CMM in PTCs. PTCs were treated with CMM or ANG II-CMM for 24 h. STAT3 phosphorylation levels (A) and translocation of STAT3 to the nucleus (B) were evaluated by Western blot analysis and immunocytochemistry, respectively. pSTAT3, phosphorylated STAT3; tSTAT3, total STAT3. Data are expressed as means ± SE. *Significant difference compared with each control consisting of untreated PTCs (P < 0.05); #significant difference compared with the ANG II-CMM-treated group (P < 0.05).
Furthermore, the high dose of ANG II did not change AT₁R levels in M/H9021, which also corresponds with a previous finding that only lower doses of ANG II increase AT₁R levels in M/H9021. Experiments using olmesartan in the present study confirmed that AT₁Rs mediate the ANG II-induced IL-6 upregulation observed in MΦ. This may explain how AT₁R blockage inhibits intrarenal AGT elevation in ANG II-infused rats (18) and spontaneously hypertensive rats (17), whereas direct treat-
ment of cultured PTCs with ANG II does not alter AGT expression levels, as shown in a previous study (37) and the present study.

CMM collected from MΦ slightly but significantly increased AGT mRNA and protein expression in PTCs. CMM collected from ANG II-treated MΦ markedly enhanced the AGT augmentation. Importantly, the enhanced AGT upregulation was attenuated by an IL-6-neutralizing antibody, indicating that the elevated IL-6 in CMM mediates stimulation of AGT expression in PTCs. The AGT augmentation by CMM and ANG II-treated CMM was not lowered to the control levels even after higher concentrations of the IL-6 antibody were used. This may be due to incomplete blockade of the receptor by the antibody, in which case the decrease in AGT after its addition would not fully represent the total effect of IL-6. It is also possible that besides IL-6, MΦ may produce another factor(s) that stimulates AGT expression in PTCs. For example, previous studies have demonstrated ROS as well as interferon-γ as factors that also contribute to intrarenal AGT augmentation (26, 39). Therefore, MΦ activated by ANG II might stimulate AGT expression in PTCs via complex mechanisms in which elevated IL-6 is the primary contributor.

The contribution of activated JAK-STAT signaling to the development of kidney injury has been established using JAK inhibitors or SOCS overexpression (1, 31, 47). Moreover, IL-6 augments AGT expression via activation of the JAK-STAT pathway in hepatocytes and PTCs (33, 37). In the present study, STAT3 phosphorylation was induced by CMM, and further activation of STAT3 was observed in PTCs that received CMM obtained from ANG II-treated MΦ. An IL-6-neutralizing antibody and a JAK2 inhibitor (AG-490) prevented the phosphorylation of STAT3. CMM-induced AGT augmentation in PTCs was also attenuated by AG-490. Interestingly, AG-490 completely diminished AGT augmentation to control levels. Additionally, SOCSs have been identified as endogenous suppressors of the JAK-STAT pathway (19). In the present study, overexpression of SOCS3 in PTCs also resulted in partial suppression of AGT augmentation stimulated by CMM obtained from ANG II-treated MΦ. Taken together, these results indicate that activation of the JAK-STAT pathway mediates CMM-induced AGT augmentation.

In conclusion, the findings in the present study suggest sequential mechanisms underlying proximal tubular AGT upregulation that occur in ANG II-dependent hypertension. In this system, elevated IL-6 production in activated MΦ might stimulate AGT expression in PTCs via complex mechanisms in which elevated IL-6 is the primary contributor.

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