Blocking of interleukin-1 suppresses angiotensin II-induced renal injury

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Abstract

Clinical hypertension is associated with renal inflammation and elevated circulating levels of proinflammatory cytokines. IL-1 receptor antagonist (IL-1Ra) is one of the most important anti-inflammatory cytokines and plays a crucial role in inflammation. Inhibition of IL-1 may contribute to modulation of the Angiotensin II (AngII)-induced hypertension response. This study aimed to elucidate the effects of IL-1Ra and anti-IL-1β antibody (01BSUR) on AngII-induced renal injury.

To determine the contribution of IL-1Ra to AngII-induced renal inflammation, male wild-type (WT) and IL-1Ra-deficient (IL-1Ra−/−) mice were infused with AngII (1000ng/kg/min) using subcutaneous osmotic pumps for 14 days. We checked renal function, histological change, and several mRNA expressions 14 days after infusion. 14 days after infusion, systolic blood pressure (197±5 vs 169±9 mmHg, p<0.05) in IL-1Ra−/− mice significantly increased compared with WT mice. Furthermore, on day 14 of AngII infusion, plasma IL-6 was 5.9-fold higher in IL-1Ra−/− versus WT mice (p<0.001); renal preproendothelin-1 mRNA expression was also significantly higher in IL-1Ra−/− mice (p<0.05). In addition, renal histology revealed greater damage in IL-1Ra−/− mice compared with WT mice 14 days after infusion. Finally, we administrated 01BSUR to both IL-1Ra−/− and WT mice, and 01BSUR treatment decreased AngII-induced hypertension and renal damage (glomerular injury and fibrosis of the tubulointerstitial area) in both IL-1Ra−/− and WT mice compared with IgG2a treatment. Inhibition of interleukin-1 decreased AngII-induced hypertension and renal damage in both IL-1Ra−/− and WT mice, suggesting suppression of IL-1 may provide an additional strategy to protect against renal damage in hypertensive patients.
Introduction

Hypertension (HT) is one of the most important causes of death worldwide. Improved treatment strategies for HT, including antihypertensive agents, have increased survival rates. However, hypertensive patients with renal injury are at increased risk for death, and improved survival of hypertensive patients potentiates the incidence of renal injury [1]. Renal injury is associated with a poor quality of life and extensive health care costs. Therefore, identifying novel therapeutics that prevent renal injury in hypertensive patients by inhibiting renal inflammation is crucial.

HT triggers a sterile inflammatory reaction, and inflammation contributes to atherosclerosis and renal injury. Proinflammatory cytokines, such as interleukin (IL)-1β [2, 3] and IL-6 [4, 5], increase as HT becomes more severe. However, the precise mechanism by which inflammation leads to HT and renal injury is poorly understood.

Angiotensin II (Ang II), the main effector of the renin-angiotensin system, increases arterial pressure and activates components of the inflammatory cascade, which promotes hypertension, endothelial dysfunction, and vascular damage [6, 7]. Both IL-1α and IL-1β are potent proinflammatory cytokines that act on endothelial cells and smooth muscle cells (SMCs) in vascular homeostasis [8]. IL-1α and IL-1β induce expression of surface leukocyte adhesion molecules in endothelial cells, proliferation of SMCs, and secretion of other cytokines and chemokines from endothelial cells, SMCs,
and macrophages [8].

IL-1 receptor antagonist (IL-1Ra), which is produced by endothelial cells, SMCs and macrophages, negatively regulates the signaling of IL-1α and IL-1β, binding and blocking the functional receptor without activation [8]. The balance between IL-1 and IL-1Ra significantly affects host responses to inflammation. Thus, IL-1Ra plays an anti-inflammatory role in acute and chronic inflammation [9]. Because inflammation is a key component in the pathogenesis of hypertension and cardiovascular disease, IL-1Ra may contribute to the modulation of Ang II-induced hypertensive response.

This study aimed to elucidate the effect of IL-1Ra on Ang II-mediated hypertension and renal injury. We also investigated whether pharmacological interference with IL-1 signaling by administration of anti-IL-1β antibody (01BSUR) could reduce Ang II-induced HT and renal injury in mice model.
Experimental

Animals

We used 10- to 12-week-old male IL-1Ra-deficient (IL-1Ra<sup>−/−</sup>, C57BL/6 background) and wild-type (WT) mice. The generation of IL-1Ra<sup>+/−</sup> mice, which lack all 4 IL-1Ra isoforms, was described previously [10]. Our studies were performed in accordance with National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All experimental procedures were approved by Juntendo University Board for Studies in Experimental Animals (No 290010). All mice were bred and housed in animal facility of our institute under a 12:12-hour light-dark cycle. Normal diet (salt content: 0.66g/100g) and water ad libitum were available during experiment.

Blood Pressure Study

Implantable mouse blood pressure transmitters (model HD-X10, Data Sciences International, St. Paul, MN) were used to directly measure blood pressure in individual mice. The devise is thought to provide the most accurate method available at present to monitor blood pressure in conscious, freely moving mice [11]. After baseline mean arterial pressure (MAP) recordings were made, hypertension was induced by Ang II (1000ng/kg per minute) infusion via a subcutaneously implanted osmotic pump (Alzet). IL-1Ra<sup>−/−</sup> and WT mice were randomly divided into 4 groups: (i) WT plus saline; (ii) IL-1Ra<sup>−/−</sup> plus saline; (iii) WT plus Ang II; and (iv) IL-1Ra<sup>−/−</sup> plus Ang II. We checked histological change, and several mRNA expressions 14 days after AngII infusion. For surgery, mice were
anesthetized with inhaled isoflurane.

**Inhibition of IL-1β by anti-IL-1β monoclonal antibody (01BSUR)**

To evaluate the effect of IL-1β on Ang II-induced HT and renal injury, both IL-1Ra−/− and WT mice were injected intraperitoneally with 7.5 mg/kg of mouse IL-1β monoclonal antibody (01BSUR) or control IgG2a twice per week after Ang II infusion. 01BSUR was gifted by Novartis Pharmaceutical and control IgG2a was purchased from Jackson ImmunoResearch.

**Inhibition of endothelin-1 (ET-1) by an endothelin receptor antagonist (bosentan)**

To evaluate the effect of ET-1 on Ang II-induced HT and renal injury, bosentan (Actelion) was mixed with powdered food supplied to IL-1Ra−/− mice at an active dose of 100mg/kg/day for 14 days as described in previous study [12].

**Suppression of blood pressure by an antihypertensive drug (hydralazine)**

To evaluate the effect of blood pressure on Ang II-induced renal injury, hydralazine (Sigma-Aldrich) was administrated at the dose 250mg/L/day in the drinking water for 14 days as described in previous report [13].
Serum IL-6 and ET-1 Levels

Serum IL-6 (GE Healthcare) and ET-1 (R&D systems) concentrations were determined by an enzyme-linked immunosorbent assay kit according to manufacturers’ instructions from blood samples after 14 days of infusion.

Renal Function Analysis

After the infusion of Ang II or saline for 14 days, twenty-four hour urine samples were collected using metabolic cages (Natume seisakujo) and also blood samples were collected. Urine albumin levels were measured by an enzymatic assay at Mitsubishi chemical medience corporation (Tokyo, Japan). The levels of urinary albumin and creatinine, and the serum levels of blood urea nitrogen (BUN) and creatinine were measured by automatic measurement at Mitsubishi chemical medience corporation (Tokyo, Japan).

Both urine and tissue kidney injury molecule-1 (KIM-1) (R&D Systems) and neutrophil gelatinase-associated lipocalin (NGAL) (R&D systems) concentrations were determined by an enzyme-linked immunosorbent assay kit according to manufacturers’ instructions from urine samples after 14 days of infusion. The supernatants of renal tissue lysates were prepared as previously reported [14]. Briefly, each kidneys were weighed and homogenized in PBS containing 1% Triton X-100, 1 mM EDTA, and 1% protease inhibitor cocktail (MilliporeSigma). Then, they were spun clear at 12,000 rpm for 10 minutes.
Quantitative Real-Time PCR

To measure mRNA expression after 14 days of infusion, we sacrificed 4–8 mice from each group. Total RNA was isolated from the samples using TRI reagent (Sigma-Aldrich). Complementary DNA (cDNA) was synthesized using reverse transcriptase from total RNA (200 ng) according to the manufacturer's protocol (High Capacity cDNA Reverse Transcription Kit; Applied Biosystems). We performed real-time PCR as described previously [15]. The following oligonucleotide primer pairs were used:

Collagen type I (forward): 5' - GTCCCAACCCCAAAGAC - 3'
Collagen type I (reverse): 5' - CATCTTCTGAGTTTGGTGATACGT - 3'
Collagen type III (forward): 5' - TGGTCCTCAGGGGTAAAGG - 3'
Collagen type III (reverse): 5' - GTCCAGCATCACCTTTG GT - 3'
IL-1α (forward): 5' - AGACCATCCAACCAGATCA - 3'
IL-1α (reverse): 5' - TGACAACTTCTGCTGACG - 3'
IL-6 (forward): 5' - ACAACCAGGCTTCCCTACTT - 3'
IL-6 (reverse): 5' - CACGATTCCCAGAGACATGTG - 3'
IL-17A (forward): 5' - CTCAGACTACCTCAACCGTTCC - 3'
IL-17A (reverse): 5' - ATGTGGTGCTCCAGCTTTCC - 3'

preproET-1 (forward): 5' - CTGCACCTCCATTCTAGCTCC - 3'
preproET-1 (reverse): 5' - TTCCCGTGATCTTCTCTTCG - 3'

TGF-β (forward): 5' - GTTGACTTTTCTCTGGTATGAGATGC - 3'
TGF-β (reverse): 5' - ACAAGGTGCCGCCGACTAC - 3'

TNF-α (forward): 5' - GGCAGGTCATCTTTGAGTCATTGC - 3'
TNF-α (reverse): 5' - ACATTCGAGGCTCCAGTGAATTGG - 3'

GAPDH (forward): 5' - AAGCCCATCACCATCTTCCA - 3'
GAPDH (reverse): 5' - GGTCAGGGTTTCTACTCC - 3'

The housekeeping gene GAPDH was used as an internal control.
Tissue Preparation and Histological Examinations

After measuring MAP, mice were killed by injection of an overdose of pentobarbital and perfused with saline followed by 4% paraformaldehyde. The kidneys were fixed in 10% formalin, embedded in paraffin, and sectioned (3 μm thickness). All samples were routinely stained with periodic acid-Schiff (PAS) to determine glomerular injury and Elastica Masson to assess tubulointerstitial fibrosis. Mesangial matrix expansion was defined by the presence of increased amounts of periodic acid-Schiff positive material in the mesangial region of each glomerulus. 25 glomeruli were selected randomly to quantify the glomerular matrix. The score was assessed by the area of PAS-positive for each glomerulus (0, normal; 1, 0-25%; 2, 25-50%; 3, 50-75%; 4, 75-100%). A final average score was calculated as a glomerular injury score [16].

To quantify the fibrosis of tubulointerstitial area, five areas of renal medulla were randomly selected. The percentage of each area that showed fibrotic changes (blue area in Elastica Masson staining) was measured by NIH Image J software version 1.47.

Immunohistochemistry

Immunostaining was done with a Discovery XT stainer (Ventana Medical Systems). After blocking endogenous peroxidase activity and revitalizing the tissue antigens with CC1 buffer, the following primary antibodies were applied: polyclonal rabbit anti-endothelin-1 (abcam) and monoclonal rat
anti-mouse Mac-3 (BD Biosciences). Antigens were visualized with the staining system, iView DAB Detection Kit (Ventana Medical Systems), and with hematoxylin counterstaining. The ratio of the positive staining area for Mac-3 to vascular area was quantified using the KS400 Carl Zeiss image analysis system (Carl Zeiss Imaging Solutions GmbH).

**Statistical Analysis**

Time-dependent changes in systolic blood pressure were analyzed with repeated-measures ANOVA. Significant F values were followed by the Dunnett test to determine specific intragroup differences and completely randomized ANOVA plus Fisher’s least significant difference test each day to determine specific intergroup differences. All measurements are shown as mean ± SEM. The results from four groups were analyzed by one-way ANOVA followed by Dunnett’s Multiple Comparison test. All statistical analyses were performed with GraphPad Prism version 5.02 (Graph Pad Software). The values of p < 0.05 were considered significant.
Results

Blood Pressure Study

On day 14 of Ang II infusion (1000ng/kg/min), plasma levels of IL-6 (605±125 (n=14) vs. 10±2 pg/mL (n=12), p<0.001)(Figure 1A). ET-1 is a key mediator of vascular constriction and kidney fibrosis [17, 18], and Ang II induces ET-1 production [19]. Renal preproendothelin-1 mRNA expression (2.6-fold, p<0.001)(Figure 1B) were significantly higher in IL-1Ra−/− mice compared with WT mice. Serum levels of ET-1 were also higher in IL-1Ra−/− mice than those in WT mice 14 days after Ang II infusion (Figure 1C).

We also monitored MAP in the Ang II-infused mice. At baseline, MAP was similar between groups (IL-1Ra−/− mice: 132±2.1 (n=8) vs. WT mice: 129±4.2 mmHg (n=8)). In both IL-1Ra−/− and WT mice, MAP increased significantly by day 2. MAP peaked by day 8 in IL-1Ra−/− mice and by day 10 in WT mice, and remained continuously high through the end of 14-day Ang II infusion in both groups (Figure 1D). We also determined that genetic deletion of IL-1Ra in mice significantly increased Ang II-induced hypertension (Figure 1D).

We compared heart/body weight (H/B) ratio in the four groups. Ang II infusion in both IL-1Ra−/− and WT mice significantly increased the H/B ratio (Figure 1E), suggesting chronic levels of blood pressure elevation. The development of cardiac hypertrophy was significantly deleterious in IL-1Ra−/− mice compared with WT mice (Figure 1E), indicating that IL-1Ra deficiency occurred at higher levels of Ang II-induced hypertension.
IL-1Ra deficiency promoted renal fibrosis in Ang II-infused mice

To evaluate the role of IL-1Ra in renal histological changes, we harvested the kidneys of Ang II or saline-infused mice 14 days after infusion. Histological analysis revealed that glomerular injury (Figure 2A: PAS staining) and tubulointerstitial fibrosis (Figure 2A: Elastica Masson staining) increased significantly in Ang II-infused IL-1Ra−/− versus Ang II-infused WT mice (Figure 2B and 2C).

IL-1Ra deficiency deteriorated renal function following Ang II infusion

To examine renal function, we analyzed 24-h urinary protein excretion (Figure 3A) and serum levels of BUN (Figure 3B), creatinine (Figure 3C), 24-h creatinine clearance (Figure 3D), and both urine and renal tissue levels of KIM-1 (Figure 3E and 3F) and NGAL (Figure 3G) in IL-1Ra−/− and WT mice. On day 14 of Ang II infusion, the levels of urinary albumin, and serum BUN and creatinine increased significantly in IL-1Ra−/− mice compared with WT mice (Figure 3A-C). Furthermore, the creatinine clearance decreased significantly in IL-1Ra−/− mice (Figure 3D). The concentrations of both urine and renal tissue KIM-1 and NGAL increased significantly in Ang II-infused IL-1Ra−/− compared with Ang II-infused WT mice (Figure 3E – 3G). RT-PCR revealed that mRNA levels of TGF-β (2.4-fold, p<0.001) in the kidneys significantly increased in IL-1Ra−/− mice compared with WT mice 14 days after Ang II infusion (Figure 3H). These findings suggest that IL-1Ra deficiency promoted renal
injury following Ang II infusion.

The mRNA expression levels of IL-1α, TNF-α, and IL-17A in AngII-treated IL-1Ra<sup>+</sup> mice were highest in four groups, but not significant statistically (Figure 4A – 4C). Regarding the profibrotic markers, mRNA levels of collagen type I in the kidneys significantly increased in IL-1Ra<sup>+</sup> mice compared with WT mice 14 days after Ang II infusion (Figure 4D). The expression pattern of collagen type III mRNA was similar to collargen type I, but not significant statistically (Figure 4E).

**IL-1Ra deficiency promoted ET-1 distribution and increased macrophage infiltration in the kidney following Ang II infusion**

The renal medulla is a main source of ET-1 [20, 21], and we detected the medulla of both Ang II-infused IL-1Ra<sup>+</sup> and WT mice had abundant ET-1 (Figure 5A). Interestingly, ET-1 was elevated in glomeruli and tubules in the cortex of IL-1Ra<sup>+</sup> mice compared with WT mice (Figure 5B), indicating elevated ET-1 might contribute to glomerular and intra-tubule fibrosis in Ang II-infused IL-1Ra<sup>+</sup> mice.

Immunostaining also revealed that Ang II-infused IL-1Ra<sup>−/−</sup> mice showed significant increased macrophages infiltration in the kidney compared with Ang II-infused WT mice (Figure 5C), suggesting that IL-1Ra deficiency increased renal inflammation following Ang II infusion.

**Treatment with an Anti-IL-1β Antibody Suppressed Ang II-induced HT and Renal Injury**
Next, we administrated 01BSUR to the mice, which significantly decreased plasma levels of IL-6 in IL-1Ra+ mice compared with IgG2a (594±103 (n=10) vs. 121±25 pg/µL (n=8), p<0.01) at 14 days after infusion. Furthermore, 01BSUR treatment reduced Ang II-induced HT (Figure 6A) at 14 days in IL-1Ra+ mice compared with IgG2a treatment. Interestingly, 01BSUR also decreased Ang II-induced HT in WT mice compared with IgG2a treatment (Figure 6A).

Histological analysis revealed that glomerular injury (Figure 6B: PAS staining) and tubulointerstitial fibrosis (Figure 6B: Elastica Masson staining) decreased significantly in 01BSUR-treated IL-1Ra+/− versus IgG2a-treated IL-1Ra+/− mice (Figure 6C and 6D). These findings suggest that 01BSUR suppresses Ang II-induced inflammation and renal injury.

Furthermore, the development of cardiac hypertrophy was significantly suppressed in 01BSUR-treated IL-1Ra+ mice compared with IgG2a-treated IL-1Ra+/− mice (Figure 6E), indicating that 01BSUR was also effective for inhibition of Ang II-induced hypertrophy in IL-1Ra+ mice.

Treatment with an Endothelin Receptor Antagonist Inhibited Ang II-induced Renal Injury in IL-1Ra+ mice, but not Hydralazine

Bosentan, an endothelin receptor antagonist, significant decreased Ang II-induced HT at 14 days in IL-1Ra+ mice (Figure 7A). The glomerular injury (Figure 7B) and tubulointerstitial fibrosis (Figure 7C) decreased significantly in bosentan-treated IL-1Ra+/− mice. On the other hand, treatment of hydralazine, an antihypertensive agent, did not suppress renal damage (Figure 7E and 7F) in Ang
II-infused IL-1Ra⁻/⁻ mice in spite of improvement of HT (Figure 7D).
Discussion

The present study demonstrates that serum IL-6 levels and MAP increased significantly in Ang II-infused IL-1Ra−/− mice compared with Ang II-infused WT mice. Previous studies have shown that Ang II stimulates the release of IL-6 [22, 23], and attenuates Ang II-induced HT in IL-6-deficient mice [5, 24], suggesting that increased levels of serum IL-6 correspond to significantly elevated blood pressure resulting from Ang II-induced inflammation. These findings are consistent with our results.

Our data also showed that IL-1Ra-deficiency increased ET-1 expression in the kidneys of Ang II-infused mice. Furthermore, serum IL-6 levels were higher in IL-1Ra−/− mice compared with WT mice after Ang II infusion. These results are consistent with an earlier report showing that IL-6 stimulates production of ET-1, and increased serum levels of ET-1 contributes to Ang II-induced HT [25]. ET-1 is a downstream mediator that causes Ang II-induced HT [26]. Thus, our findings show that deficiency of endogenous IL-1Ra promotes kidney production of ET-1 and serum levels of ET-1 after Ang II infusion, contributing in turn to Ang II-induced HT. This is the first study to demonstrate that IL-1Ra contributes to the suppression of Ang II-induced hypertension by inhibiting ET-1 elevation. Furthermore, we show that 01BSUR treatment significantly decreases plasma levels of IL-6, similar to a previous clinical study [27], and reduces ET-1 mRNA expression in Ang II-infused IL-1Ra−/− mice, resulting in decreased blood pressure at 14 days after infusion.

Our data showed that IL-1Ra-deficiency increased ET-1 expression in the kidneys of Ang II-infused mice and promoted renal fibrosis. In this study, we detected expression of ET-1 protein was elevated in glomeruli and tubules in the cortex of IL-1Ra−/− mice compared with WT mice at 14 days.
after Ang II infusion. Previous reports demonstrated that high intra-glomerular ET-1 expression has been implicated in structural damage, leading to glomerulosclerosis [28] and ET-1 overexpression in transgenic mice caused glomerulosclerosis [29]. These findings suggest that expression of ET-1 in glomerular and tubular should contribute to progression of their fibrosis in Ang II-infused IL-1Ra−/− mice. RT-PCR revealed that mRNA levels of TGF-β in the kidneys significantly increased in IL-1Ra−/− mice compared with WT mice. It is also possible that ET-1 promoted fibrosis by elevating expression of TGF-β, although ET-1 can contribute directly to raise collagen type I production in mesangial cells [30]. Actually, RT-PCR demonstrated that mRNA levels of collagen type I in the kidneys significantly increased in Ang II-infused IL-1Ra−/− mice. Thus, our findings show that deficiency of endogenous IL-1Ra promoted kidney production of ET-1, collagen type I, and TGF-β after Ang II infusion, contributing in turn to glomerular and intra-tubular fibrosis.

IL-1Ra deficiency accelerated Ang II-induced renal injury, and inhibition of IL-1β using 01BSUR suppressed Ang II-induced renal injury, suggesting an important role for IL-1β during the progression of Ang II-induced renal injury. IL-1 consists of IL-1α and IL-1β, both of which exert a similar, but not completely same biological mediated through the IL-1 type I receptor [9]. IL-1Ra binds to IL-1 receptors without exerting agonistic activity and negatively regulates the signaling of IL-1α and IL-1β [9, 10]. Suppression of IL-1β with 01BSUR significantly inhibited renal injury in Ang II-treated both IL-1Ra−/− and WT mice, indicating that IL-1β acts predominantly on renal injury in Ang II-treated mice. Previous study showed that glomerular expression of IL-1β has been
demonstrated in proliferative forms of human glomerulonephritis [31]. Furthermore, an experimental study revealed IL-1β deficiency reduced cellular mediators of glomerular injury and attenuated glomerulonephritis independent of IL-1α in murine model [32]. These findings support our results. Taken together, the suppression of IL-1β ameliorated Ang II-induced renal injury.

Canakinumab, a human monoclonal antibody targeting IL-1β, is approved for treatment of cryopyrin-associated periodic syndrome (CAPS) in humans [33]. In our study, 01BSUR significantly decreased Ang II-induced renal injury in both WT and IL-1Ra−/− mice compared with IgG2a treatment, suggesting that canakinumab might be useful not only for CASP, but also for organ injury. Indeed, the CANTOS (Canakinumab Anti-Inflammatory Thrombosis Outcomes Study) demonstrated that canakinumab reduces major adverse cardiovascular event rates among patients with prior heart attack and inflammatory atherosclerosis [34]. Furthermore, subanalysis of the CANTOS revealed that canakinumab significantly reduced cardiovascular event rates among atherosclerosis patients with chronic kidney disease and has no substantive adverse effects on indices of renal function over time [35]. Moreover, our study suggests that early initiation of canakinumab might suppress Ang II-induced renal injury without any adverse effects.

Finally, we investigated whether pharmacological inhibition of ET receptors could prevent Ang II-induced HT and renal injury in IL-1Ra−/− mice. We found bosentan treatment significantly decreased Ang II-induced HT at 14 days in IL-1Ra−/− mice, and there was no significant difference in the blood pressure between bosentan treated and 01BSUR treated IL-1Ra−/− mice (172.2±7.3 vs
162.2±15.3 mmHg, p=0.57). These findings indicate ET-1 synthesis and its vasoconstrictive effect should contribute to the elevated blood pressure in IL-1Ra⁻/⁻ mice. Furthermore, treatment of bosentan protected Ang II-infused IL-1Ra⁻/⁻ mice against renal injury. On the other hand, treatment of hydralazine, an antihypertensive agent, did not suppress renal damage in Ang II-infused IL-1Ra⁻/⁻ mice in spite of improvement of HT. This results suggest that the anti-inflammatory action of bosentan [12] may be direct or indirect through protective effects against Ang II-induced renal injury in IL-1Ra⁻/⁻ mice beyond an anti-hypertensive therapy.

Figure 8 summarizes our findings. Lack of IL-1Ra enhanced the effect of IL-1β, and IL-1β augmented inflammation and we detected many macrophages in the kidneys from Ang II-infused IL-1Ra⁻/⁻ mice. Then increased cytokine production (IL-6) resulted in imposing several changes. (i) Increased levels of IL-6 contributed to Ang II-medicated induction of ET-1 production, and increased circulating ET-1 should increase in blood pressure; (ii) proinflammatory cytokines and ET-1 activated several TGF-β that promoted fibrotic changes in the kidney; (iii) TGF-β and ET-1 promoted tubular fibrosis and glomerular damage. These changes should aggravate renal injury. Our findings strongly suggest that blocking IL-1β therapeutically target HT-induced renal injury.

There are several limitations in this study. First, we used relatively young mice (10- to 12-week-old) in this study. The older mice could not show the significant responses of 01BSUR for the suppression of both HT and renal injury. Second, we used the mice without atherosclerosis, resulting in the significant effect of 01BSUR on blood pressure in our study. Finally, there are a plurality of
influencing factors for causing HT in human, but we only used Ang II-induced HT model mice.

Although there are several limitation shown above, we believe our findings suggest that early initiation of an anti-IL-1β monoclonal antibody might suppress Ang II-induced renal injury without any adverse effects. Disruption of IL-1β signaling deserves further evaluation in large animal models or patient trials for the suppression of HT-induced renal injury.

In conclusion, we demonstrate here that IL-1β inhibition decreases Ang II-induced HT and suppresses renal injury in mice model, suggesting that such inhibition could provide an additional strategy to protect against chronic kidney dysfunction in hypertensive patients.
Clinical perspectives

· Background as to why the study was undertaken

Clinical hypertension is associated with renal inflammation and elevated circulating levels of proinflammatory cytokines. IL-1 receptor antagonist (IL-1Ra) is one of the most important anti-inflammatory cytokines and plays a crucial role in inflammation. Inhibition of IL-1 may contribute to modulation of the Angiotensin II (AngII)-induced hypertension response. Our primary question was which is true, that is, blockade of IL-1 signaling by endogenous IL-1Ra or anti-IL-1β antibody (01BSUR) is effective or not, for AngII-induced renal injury.

· A brief summary of results

We found that inhibition of IL-1 by either endogenous IL-1Ra or exogenous 01BSUR decreased AngII-induced hypertension and renal damage in mice.

· The potential significance of the results to human health and disease

We demonstrated that IL-1β inhibition decreases Ang II-induced hypertension and suppresses renal inflammation in mice model, suggesting that early initiation of anti-IL-1β antibody (canakinumab) might suppress Ang II-induced renal injury without any adverse effects.

Data Availability

The authors confirm that the data supporting the findings of this study are available within the article, its supplementary materials and from the corresponding author [K.I.] upon reasonable
request.

**Competing Interests**

None declared.

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**Abbreviations**

Ang II, angiotension II; ET-1, endothelin-1; H/B ratio, heart/body weight ratio; HT, hypertension; IL, interleukin; IL-1Ra, interleukin-1 receptor antagonist; MAP, mean arterial pressure; SMC, smooth muscle cells; TGF, transforming growth factor; WT, wild-type.
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Figure Legends

Figure 1

IL-1Ra deficiency promoted Ang II-induced hypertension and aortic inflammation. Serum levels of IL-6 (A), renal preproendothelin-1 (ET-1) mRNA expression (B), and serum levels of ET-1 (C) were increased significantly in Ang II-infused IL-1Ra-deficient mice compared with other three groups at 14 days after Ang II infusion. WT: wild-type mice, Ra-/−: IL-1Ra-deficient mice, NS: normal saline, Ang II: angiotensin II. *p<0.05, **p<0.01, ***p<0.001, n=8-12 per group.

(D) Genetic deletion of IL-1Ra in mice significantly increased Ang II-induced mean arterial pressure. *p<0.05 vs WT Ang II; * † p<0.05 vs Ra-/− NS; #p<0.05 vs WT NS, n=4-6 per group.

(E) heart/body weight (H/B) ratio was increased significantly in Ang II-infused IL-1Ra-deficient mice compared with other three groups at 14 days after Ang II infusion.

**p<0.01, ***p<0.001, n=6-9 per group.

Figure 2

Deficiency of IL-1Ra promoted glomerular injury and renal fibrosis in Ang II-infused mice.

A, Microscopic appearances for Periodic acid-Schiff (PAS) (upper panels, scale bars=25μm) and elastica Masson (lower panels, scale bars=50μm) staining in the kidneys from 4 groups of mice 14 days after Ang II or normal saline (NS) infusion. Renal histological findings in Ang II-infused IL-1Ra+/− mice changed significantly compared with Ang II-infused WT mice. The glomerular injury
score (B) and fibrosis of tubulointerstitial areas (C) increased significantly in Ang II-infused IL-1Ra⁻/⁻ mice compared with Ang II-infused WT mice. **p<0.01, ***p<0.001; n=6 per group.

Figure 3
IL-1Ra deficiency deteriorated renal function in Ang II -infused mice.

Urinary protein excretion/serum creatinine ratio (A), and serum levels of BUN (B) and creatinine (C) were significantly higher in IL-1Ra⁻/⁻ mice than in WT mice 14 days after Ang II infusion. The creatinine clearance (CCr) (D) decreased in IL-1Ra⁻/⁻ mice significantly. The concentrations of urine kidney injury molecule-1 (KIM-1) (E), KIM-1 levels in kidney tissues (F) and neutrophil gelatinase-associated lipocalin (NGAL) (G) increased significantly in IL-1Ra⁻/⁻ mice. RT-PCR revealed TGF-β mRNA (H) increased significantly in IL-1Ra⁻/⁻ mice. *p < 0.05, **p < 0.01, ***p < 0.001; n = 5–7 per group.

Figure 4
Expression of genes involved in cytokines, and profibrotic markers in the kidneys of IL-1Ra⁻/⁻ and WT mice.

The mRNA expression levels of IL-1α (A), TNF-α (B), and IL-17A (C) in AngII-treated IL-1Ra⁻/⁻ mice were highest in four groups, but not significant statistically. Regarding the profibrotic markers, mRNA levels of collagen type I in the kidneys significantly increased in IL-1Ra⁻/⁻ mice compared with WT mice 14 days after Ang II infusion (D), but not collagen type III (E). *p < 0.05; n = 4 per group.
Figure 5

IL-1Ra deficiency increased ET-1 expression in glomeruli and tubules in the cortex, and kidney inflammation in Ang II-infused mice.

Microscopic appearances for ET-1 staining in the medulla (A, scale bars=500μm) and cortex (B, scale bars=100μm) in the kidneys from 2 groups of mice 14 days after Ang II infusion.

C, Representative immunohistochemistry images of macrophages (Mac3) from WT (left) and IL-1Ra-/- (middle) mice 14 days after Ang II infusion (scale bars=100μm). Macrophage staining was quantified as the percentage of total kidney area that was stained (right).

Figure 6

Treatment with an anti-IL-1β antibody (01BSUR) suppressed Ang II-induced HT and renal injury.

A, An anti-IL-1β antibody (01BSUR) suppresses Ang II-induced hypertension in both WT (left) and IL-1Ra-/- mice (right).

B, Microscopic appearance for Periodic acid-Schiff (PAS) (upper panels, scale bars=25μm) and elastic Masson (lower panels, scale bars=50μm) staining in the kidneys 14 days after Ang II infusion. The glomerular injury score (C) and fibrosis of tubulointerstitial areas (D) decreased significantly in 01BSUR-treated mice compared with IgG treatment at 14 days after Ang II infusion (n=8 for each group).

E, The development of cardiac hypertrophy was significantly inhibited in 01BSUR-treated IL-1Ra-/- mice compared with IgG2a-treated IL-1Ra-/- mice at 14 days after Ang II infusion (n=5 for each group).
Data are expressed as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001.

**Figure 7**

Treatment with an endothelin receptor antagonist (bosentan) inhibited Ang II-induced renal injury in IL-1Ra−/− mice, but not an antihypertensive agent (hydralazine).

**A**. An endothelin receptor antagonist (bosentan) suppresses Ang II-induced hypertension IL-1Ra−/− mice.

The glomerular injury score (B) and fibrosis of tubulointerstitial areas (C) decreased significantly in bosentan-treated mice compared with control at 14 days after Ang II infusion (n=5-6).

On the other hand, treatment of hydralazine, an antihypertensive agent, did not suppress renal damage (E and F) in Ang II-infused IL-1Ra−/− mice in spite of improvement of HT (D) (n=8 for each group).

Data are expressed as mean ± SEM. *P<0.05, **P<0.01.

**Figure 8**

A model depicting the influence of IL-1Ra deficiency in Ang II induced hypertension and renal injury.
Figure 1

A. Serum IL-6

B. ppET-1 mRNA

C. Serum ET-1 Level

D. Blood pressure (mmHg)

E. H/B ratio

* p<0.05 vs WT AngII
† p<0.05 vs Ra-/ AngII
# p<0.05 vs WT NS

*** p<0.001
* p<0.05
Figure 2

A

|                | WT NS | Ra-/− NS | WT AngII | Ra-/− AngII |
|----------------|-------|----------|----------|-------------|
| PAS            | ![Image](URL) | ![Image](URL) | ![Image](URL) | ![Image](URL) |
| Bars=25µm     |       |          |          |             |
| Elastica Masson| ![Image](URL) | ![Image](URL) | ![Image](URL) | ![Image](URL) |
| Bars=50µm     |       |          |          |             |

B

**Glomerular damage**

- WT NS
- Ra-/− NS
- WT AngII
- Ra-/− AngII

**Tubulointerstitial fibrosis**

- WT NS
- Ra-/− NS
- WT AngII
- Ra-/− AngII

**p<0.01**

**p<0.05**

**NS**

![Image](URL)
Figure 3
Figure 4

(A) IL-1α

(B) TNF-α

(C) IL-17A

(D) Collagen type I

(E) Collagen type III

*p < 0.05
Figure 5
Figure 6
Figure 7

A) Graph showing blood pressure (mmHg) over days for control and bosentan groups.

B) Graph showing glomerular damage scores for Ra−/− control and Ra−/− bosentan groups.

C) Graph showing tubulointerstitial fibrosis area/field (%) for Ra−/− control and Ra−/− bosentan groups.

D) Graph showing blood pressure (mmHg) over days for control and hydralazine groups.

E) Graph showing glomerular damage scores for Ra−/− control and Ra−/− hydralazine groups.

F) Graph showing tubulointerstitial fibrosis area/field (%) for Ra−/− control and Ra−/− hydralazine groups.

*p ≤ 0.05

**p ≤ 0.0001

NS = Not Significant
Figure 8