Renin-Dependent Hypertension in Mice Requires the NLRP3-Inflammasome

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Abstract

Objective: Recent studies have implicated an enhanced secretion of IL1β through activation of the Nod-like receptor family, pyrin domain containing 3 (Nlrp3)-inflammasome as the pro-inflammatory signal in animal models of the gout, chronic kidney disease (CKD), atherosclerosis, metabolic syndrome, type 2 diabetes mellitus, but its contribution to hypertension is not established. We aimed to demonstrate the role of the Nlrp3-inflammasome in the two-kidneys, one clip (2K1C) Goldblatt model of hypertension in Nlrp3/- and apoptosis-associated speck-like protein containing a caspase recruitment domain (Asc)-/- and wild type control male mice.

Study design: 2K1C hypertension was generated by narrowing left renal artery using U-shaped stainless steel clip. BP and heart rate were recorded intra-arterially with a computerized data-acquisition system in conscious mice. Plasma renin activity and concentration were measured by radioimmunoassay. Renal transcript levels of Nlrp3, Asc, Casp1, IL1A, IL1β, and IL6 were assessed by RT-Q-PCR.

Results: Results show that Nlrp3/- and Asc/- mice are protected from developing hypertension and have lower circulating levels of plasma renin and serum amyloid A (SAA) and IL6, in comparison to wild type (WT) control mice. RNA levels of SAA, Nlrp3, and IL1β are increased in the ischemic kidney of the WT control mice. Administration of anti-IL1β antibody to the WT control mice attenuated the increase of blood pressure and renin in the 2K1C model. The results suggest that NALP3 inflammasome has an impact on blood pressure and renin secretion during renal hyperperfusion induced by renal arterial clip and contributes to renin-dependent renovascular hypertension.

Conclusion: These findings show that Nlrp3-inflammasome mediated production of IL1β is linked to the development of renovascular hypertension and might be a novel target for the treatment of renovascular hypertension, CKD and other inflammatory diseases with hypertension.

Keywords: Nlrp3-inflammasome; 2K1C hypertension; Plasma renin activity; IL-1β

Introduction

Hypertension affects approximately 25% of the adult population worldwide, and its prevalence is predicted to increase by 60% by 2025, when a total of 1.56 billion people may be affected [1]. It is the most important risk factor leading to cardiovascular diseases. About 62% of stroke and 49% of coronary heart disease are attributable to hypertension and atherosclerosis [2]. Pro-inflammatory cytokines produced by infiltrating macrophages and lymphocytes in the atherosclerotic plaque account for the pathological changes in atherosclerotic vessels and in epidemiological studies, increased levels of the inflammatory markers IL6 and C-reactive protein (CRP) are associated with a higher risk of developing cardiovascular disease [3,4]. Four inflammasomes have been identified so far and the NLR family, pyrin domain-containing 3 (Nlrp3) inflammasome has been the most fully characterized and forms multi-protein complex with apoptosis-associated speck-like protein containing a caspase recruitment domain (Asc) and the protease Caspase-1, which activates the cytokines pro-interleukin-1β (IL-1β) and pro-IL-18 [3,5] triggering inflammation. A large range of stimuli can activate this complex, ranging from pathogen associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) such as uric acid/monosodium urate (MSU)/ crystals [7], fatty acids [8], cholesterol crystals [9], K+ efflux [10] etc. Recent evidence suggests that activation of the Nlrp3-inflammasome is a potential mechanism linking inflammation to the gout [7,11], atherosclerosis [3,9], chronic kidney disease (CKD) [12,13], metabolic syndrome [14,36], obesity and type-2 diabetes mellitus [16] these frequently are accompanied with hypertension, but a direct link between Nlrp3-inflammasome and hypertension is unclear though there is increasing evidence that cytokines have a role in the development of hypertension. It has been reported that patients with essential hypertension had increased circulating IL-1β levels before any drug therapy [17]. In man, essential hypertension has been found to be associated with polymorphisms linked to the Nlrp3 gene in Japanese subjects [18], though the mechanism of this effect is unknown.

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Inflammasome-regulated cytokines such as IL-1β and IL-18 are implicated in CKD. Recent animal studies have demonstrated the role of Nlrp3-inflammasome in the development of renal inflammation [13]. Mice deficient in the components of the inflammasome showed reduced signs of renal damage in models of acute and chronic kidney disease [12]. Previous studies have demonstrated that IL1β increases plasma renin activity (PRA), an indirect indicator of both systemic activity of renin-angiotensin system, RAS, and plasma Angiotensin-II (concentration) and blood pressure [17, 9, 19]. Although IL-1β and IL-18 are known to be elevated in hypertensive patients, no studies have examined if this occurs downstream of inflammasome activation or if inhibition of inflammasome and/or IL-1β/IL-18 signaling prevents hypertension [20-22].

Renovascular hypertension (RVH) is a common public health problem, particularly in older patients with underlying atherosclerotic vascular disease. The prevalence of RVH is 7% in patients over 65 years of age [18]. It is well recognized that reduced blood flow leads to intra-renal activation of the RAS, leading to elevated plasma levels of angiotensin II, a potent vasoconstrictor, and the development of systemic hypertension. Recent evidence indicates that restoration of blood flow through stenting does not improve renal or cardiovascular outcomes, compared to medical therapy [24]. New approaches to the treatment of RVH due to RAS depend on better understanding of basic mechanisms underlying the development of chronic renal disease in these patients. The two kidney 1 clip (2K1C) model of RVH was initially invented by Goldbatt and has been extensively employed to understand the pathogenesis of RVH [25]. This model is driven by increased renin excretion during renal blood flow hypoperfusion induced by a renal arterial clip, leading to hypertension and cardiac & renal remodeling and lesions [26]. In the present study, therefore, we investigated the role of the Nlrp3-inflammasome and IL-1β in the development of rennin-dependent RVH in two-kidney, one clip (2K1C) treated Nlrp3/Asc deficiency and wild type (WT) mice.

Animals and Methods

Nlrp3-inflammasome deficient mice and 2K1C hypertension

Nlrp3-inflammasome deficient (Nlrp3-/- and Asc-/-) male mice backcrossed on the C57Bl/6J for more than 10 generations were derived from Jürg Tschopp’s group at Institute of Biochemistry of Lausanne University in Switzerland [7,27] and were used at 6-8 weeks of age. The experiment protocols were reviewed and approved by our institutional animal experimentation committee. Mice were housed in a pathogen-free facility kept on standard light (12:12-h light-dark cycle) and regular diet. 2K1C hypertension was induced by placing a U-shaped stainless steel clip (Ø=0.12 mm, Exidel S.A., Switzerland) on the left renal artery in 6-week old male mice under anaesthesia (1-2% isoflurane mixed with oxygen). The right carotid artery was exposed for a length of approximately 4 mm. A PE-10 catheter filled with 0.9% NaCl solution containing heparin (300 IU/ml) was inserted into the artery. After ligation, the catheter was subcutaneously tunneled to exit at the back of the neck. The mouse was allowed 3-hour to recover from the anesthesia and placed in a Plexiglas tube for partial restriction of their movements. Thirty minutes later, the arterial line was connected to a pressure transducer; BP and heart rate (HR) were then monitored every 20s for 15 to 30 min by Notocord HEM 3.1 software (SA, Croissy, France) at a sampling rate of 500 Hz.

After the BP measurements, 300 µL of blood was collected through the catheter into 0.6ml Multivette EDTA tube at 4°C and 300µL of blood, drawn into 0.6 ml Multivette tube containing gel/clot activator. Plasma and serum samples were frozen in liquid nitrogen and stored at -20°C for plasma renin and soluble inflammatory markers analysis. After sacrifice, the heart and kidney were excised and weighed. Half of the heart was fixed in 3.8% formalin and embedded in paraffin, another half, frozen in nitrogen and stored at -80°C for molecular and morphological examinations. Cardiac and renal hypertrophy was determined by heart and kidney indices (heart or kidney weight/body weight, mg/g). Plasma renin activity and concentration (PRA and PRC) were measured using previously described methods [25,28,29].

Nlrp3 inflammasome and inflammation marker examinations

Transcript levels of Nlrp3, Asc, Casp1, IL1α, IL1β, and IL6 were assessed by RT-Q-PCR in clipped and non-clipped kidneys (pools of at least 4 mice per group) of WT mice using Gapdh and Tbp as reference genes. Briefly, RNA was extracted from kidney tissue using Tissue Lyzer (Qiagen) and ZR-96 RNA clean and concentrator kit (ZymoResearch). Purified RNA was reverse transcribed, using Superscript II (Invitrogen). Transcript quantification was performed using SYBR Master I and gene specific primers on the Light Cycler LC480 system (Roche). Gene specific sequences were, IL1α: 5' - AAA CAC TAT CTC AGC ACC ACT TG-3', and 5'-GGT CGG TCT CAT TAC CTG TG-3'; IL1β: 5'-CCA CCA ACA AGT GAT ATT CTC CAT G-3', and 5'-GTG CGG TCT AAC ATT ACA CAG-3'; Casp1: 5'- CGG TGG AGA GAA ACA GTG TGA TGC GA-3', and 5'-GGT CCG TCT CAC TAC CTG TG-3'; IL1B: 5'- CGG TGG AGA GAA ACA GTG TGA TGC GA-3', Asc: 5'- CAG CCA AGA CAC AGT AAC CGT CTC GAG-3', and 5'- CTG GAC TTC TGA GAC TGA TGC ATC -3'; Nlrp3: 5'- TCA GTG TGT TTT CCC AGA CA-3', and 5'- GGT TGG TGC TTA GAC TG-3'; Asc: 5'- CAG CCA AGA CAC AGT AAC CGT CTC GAG-3', and 5'- CGG TGG AGA GAA ACA GTG TGA TGC GA-3'; Gapdh 5' - CTC ATG ACC ACA GTC CAT GC-3', and 5'- CAC ATT GGG GTG AGG AAC AC-3', and Tbp 5' - CTT GAA ATC ATC CTT CGG AG-3', and 5'- CGC TTT CAT TAA ATT GGT GGT C-3'. Results were calculated using the 2^ΔΔCT method, and normalized against the WT-sham mice. Serum IL6 and serum amyloid A (SAA) were measured by Elisa (eBioscience, and Biosource), according to the manufacturers’ protocols.

IL-1β Antibody Administration

200 µg of anti-mouse IL-1β antibody was injected intraperitoneally to 2K1C and sham treated C57Bl/6J male mice weekly for 6 weeks. In the control groups, 2K1C and sham mice received only saline. In the end of the experiment, BP, PRA, heart and kidney indices were measured.

Statistical analysis

Data are presented as mean ± SEM. Two-way ANOVA analysis was used to statistically evaluate differences between genotypes and treatments using GraphPad PRISM 6. P<0.05 was considered the minimal level of significance.
Results

The Nlrp3-inflammasome contributes to renin-dependent hypertension

To assess the role of Nlrp3-inflammasome in the development of renin-dependent hypertension, Nlrp3-/- and Asc-/- mice and wild type (WT) control mice were applied in 2K1C model of hypertension. (Figure 1A, 1B, 1C) shows that in WT mice, mean blood pressure, PRA, and kidney weight to body weight index (KWI) are significantly increased at week 12 after renal artery clamping. Cardiac hypertrophy is also observed in response to the increase in blood pressure (Table 1). Heart weight to body weight (HW/BW) ratio was increased to 5.3 ± 0.1 mg/g in 2K-1C versus sham-operated animals (HW/BW: 4.2 ± 0.1 mg/g, p<0.01). Neither Nlrp3-/- nor Asc-/- mice developed hypertension after the renal artery clipping. PRA and PRC were not raised by the 2K1C procedure in Nlrp3-/- and Asc-/- mice although clamping of the renal artery lead to a comparable renal ischemia and reduction of kidney weight on the clipped kidney in all three groups of mice indicating that the renal arterial clamping had a comparable effect on renal blood perfusion among 2K1C treated animals (Figure 1C and Table 1). This change in kidney weight served as an internal control that the clipping did indeed cause renal ischemia in all groups of mice, yet only the WT mice developed significant higher PRA, PRC, hypertension, and hypertrophy in unclipped kidney (Table 1 and Figure 1C). However, we did not observe hypertrophy in contralateral unclipped kidney in Nlrp3-/- or Asc-/- mice.

Renal ischemia upregulates pro-inflammatory and Nlrp3-inflammasome gene expression

The previous results suggest that renal ischemia may have an inflammatory effect. We investigated the effects of the ischemia on the expression of pro-inflammatory genes in the kidney tissue of WT mice with regard to the effects on the NLRP3-inflammasome pathway. Real-time quantitative PCR (RT-Q-PCR) was performed on pooled kidney tissues of WT mice (Figure 2). We found significant upregulation of RNA levels of Nlrp3, IL-1β and IL-1α. Only IL1β levels were unregulated in the non-clipped kidney while no significant modulation of casp1, Asc and IL6 mRNA levels were observed. These results confirmed that ischemia unregulated local transcription of the Nlrp3 gene in the clipped kidney, as well as increased expression of IL1β and IL1α mRNA. We also found a significant increase in the acute phase reactant SAA in WT 2K1C mice (Figure 1F). But, Nlrp3-/- mice did not show a rise in SAA after the procedure. RNA levels of the IL6 gene were not unregulated following ischemia in WT mice and we did not find significant changes in serum IL6 levels in either the WT and Nlrp3-/- mice following 2K1C (as compared to sham controls).

Histologically, there were no evidence of inflammatory action, fibrosis or tissue necrosis in the 12-week clipped or unclipped kidneys in WT and Nlrp3-/- mice examined by pathologist (Figure 3).
Table 1: Pathophysiological responses to 2K1C renal ischemia in WT, Nlrp3-/-, and Asc-/- mice

<table>
<thead>
<tr>
<th></th>
<th>WT-sham</th>
<th>WT-2K1C</th>
<th>Nlrp3KO-sham</th>
<th>Nlrp3KO-2K1C</th>
<th>Asc KO-sham</th>
<th>Asc KO-2K1C</th>
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<tr>
<td>MBP, mmHg</td>
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<td>146 ± 3</td>
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<td>***</td>
<td>119 ± 4</td>
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<td>Body weight (BW), g</td>
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<td>30 ± 1</td>
<td>30 ± 1</td>
<td>30 ± 1</td>
<td>30 ± 1</td>
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<tr>
<td>Heart weight (HW), mg</td>
<td>126 ± 3</td>
<td>160 ± 5</td>
<td></td>
<td>***</td>
<td>124 ± 3</td>
<td></td>
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<tr>
<td>BW/BW, g</td>
<td>4.2 ± 0.1</td>
<td>5.3 ± 0.1</td>
<td></td>
<td>***</td>
<td>4.2 ± 0.1</td>
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<tr>
<td>Heart weight (HW), mg</td>
<td>164 ± 4</td>
<td>156 ± 2</td>
<td></td>
<td>***</td>
<td>164 ± 3</td>
<td>147 ± 4</td>
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<tr>
<td>HW/BW, mg</td>
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<td>***</td>
<td>168 ± 5</td>
<td>182 ± 5</td>
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<tr>
<td>PRC, ng/ml/hr</td>
<td>559 ± 117</td>
<td>1780 ± 200</td>
<td></td>
<td>***</td>
<td>852 ± 240</td>
<td></td>
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<tr>
<td>Serum IL6, pg/ml</td>
<td>140 ± 18</td>
<td>162 ± 21</td>
<td>91 ± 18</td>
<td>ND</td>
<td>ND</td>
<td></td>
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Results are expressed as mean ± SEM, n=6-10 per group, significance determined at p≤0.05 by 2-way ANOVA analysis. *** p<0.001, 2K1C vs. sham; # # p<0.01, clipped (Left) KW vs. unclipped (Right) KW; †p<0.05, ††p<0.01, ††† p<0.001, KO vs. WT. LKW: left or clipped kidney weight. RKW: right or unclipped kidney weight. PRC: plasma renin concentration. ND: not determined.

Figure 2: Proinflammatory gene expression in renal tissue of 2K1C mice.
Transcript levels were assessed by RT-Q-PCR on pooled kidney samples (n≥3) and compared to expression in WT-sham operated mice. Results are expressed as mean ± SEM, and significance determined at * p≤0.05, ** p≤0.01, *** p≤0.001 by the Tukey’s One way Anova.

Figure 3: Histology in 12-week clipped and unclipped kidneys in WT and Nlrp3 KO mice by HE staining with 100x magnification. LK & RK: left kidney and right kidney.
to demonstrate active p17 IL1β in the clipped kidney by western blotting were unsuccessful because of the low levels of IL1β in renal tissue. However, we were able to measure total IL1β levels, including both pro-IL1β (p35) as well as the active p17 form, from kidney tissue lysates by ELISA. The mean level in sham-operated mice (n=7) was 94 ng/ml of total IL1β, compared to a mean value of 85 ng/ml in 2K1C kidneys (n=6, p<0.05). These results indicate that there was no overall change in total IL1β levels, but do not exclude an increase in active p17. These results indicate that renal ischemia induced pro-inflammatory gene transcription including RNA for Nlrp3, IL1b, and IL1a, and was associated with the development of hypertension.

### Inhibition of IL1β blocks development of 2K1C-hypertension

In order to demonstrate role of the IL1β in the development of hypertension in the 2K1C model, we investigated the effects of IL1β inhibition using a monoclonal antibody specific for IL1β. 200 μg of anti-mIL1β intraperitoneally weekly for 6 weeks in 2K1C and sham operated WT mice (5 and 6 per group respectively). The treatment prevented the rise of PRA and BP in 2K1C mice and had no effect on PRA and BP in sham animals, suggesting that IL1β secretion may participate in the regulation of renin secretion and the development of hypertension, and explains why Nlrp3-/- and Asc-/- mice did not develop hypertension in the 2K1C model.

### Discussion

Our results show for the first time that in renovascular hypertension, Nlrp3-inflammasome play a key part in the activation of the renin-angiotensin and hence in the increase in systemic blood pressure. This provides new evidence that factors other than purely hemodynamic change contribute to the pathophysiology of renovascular hypertension. The secretion of IL1β due to activation of the inflammasome results in increased levels of the acute phase reactant SAA as well as IL6 in the serum, and the absence of Nlrp3 and Asc abrogated these changes. What are the triggers of inflammasome activation in this model? Recent data showed that the Nlrp3-inflammasome could be activated by a large variety of PAMPs and DAMPs, and raise the question if there is a common final pathway that mediates these effects. The generation of ROS as a result of cell stress, the activation of the P2X7 receptor by ATP binding and the intracellular release of cathepsins have all been proposed as intermediate steps in inflammasome activation [30]. In our model, the most plausible pathway is the generation of ROS following ischemia/reperfusion of the clipped kidney that acts as the trigger. Recent data have implicated the Nlrp3-inflammasome in the reaction to ischemia-reperfusion injury in the myocardium and the liver [31,32]. In the 2K1C model, although there was no significant tissue inflammatory infiltration of the clipped or the contralateral unclipped kidney by standard histological examination, the presence of a weight index difference between the two kidneys is evidence that there was significant ischemia, which was sufficient to induce inflammasome activation and IL1β release. We have not determined the exact tissue origin of IL1β in the current studies, but it is likely that the secretion originates from monocytes/macrophage lineage cells and from vascular endothelial cells, as previously demonstrated in rats [33].

The local release of IL1β enhances the secretion of renin and activation of the renin-angiotensin system, resulting in hypertension as well as the amplification of local IL1 levels with increased IL1α transcription, as demonstrated by the QT-PCR results. Moreover, angiotensin II has been shown to activate monocytes thereby contributing to a further amplification of the inflammatory process [34]. The absence of hypertrophy in the heart and in the contralateral unclipped kidney in Nlrp3-/- and Asc-/- 2K1C treated mice could be due to abolishment of angiotensin-II (Ang-II) mediated nonhemodynamic effects including promoting fibrosis by stimulation of TGF-β receptor type-II and up-regulating expression and synthesis of NF-kB and other cytokines, absence of hypertension, or block the inflammasome mediated inflammation response. However, the mechanisms underlying the differential response of the stenotic and contralateral kidney during the development and progression of RVH have not been adequately defined, there should be careful consideration and evaluation of the role of the immunologic and cytokine-associated effects of RAS in the pathological process and initiation of RAS induced kidney damage.

The 2K1C model reproduces many of the clinical and physiological features of renovascular hypertension in man. Although pure RVH accounts for only a small proportion of patients with hypertension, renovascular disease is common in patients with atherosclerosis, and its incidence is increasing in the aging population [18,35]. Until now, management of renal artery stenosis was predicated on restoration of blood flow to the stenotic kidney. It was thought that this intervention would decrease local and systemic activation of the renin-angiotensin system, thereby restoring normal blood pressure and reducing both renal and cardiovascular morbidity and mortality. But, recent evidence indicates that restoration of blood flow alone does not improve renal or cardiovascular outcomes in patients with renal artery stenosis [24]. Our results suggest that inhibitors of inflammation may be of benefit in such a situation at least by reducing the RAS activity and BP in patients with atherosclerotic renal artery disease, diabetic nephropathy, and other CKD, but the currently used inhibitors, such as NSAIADs or corticosteroids would pose problems for patients with impaired renal function or poorly controlled hypertension. In this context, IL1 inhibition may be an interesting therapeutic alternative. IL1 inhibition has been found to be extremely effective in the treatment of monogenic autoinflammatory disorders due to mutations of the Nlrp3 gene [30] in the treatment of MSU-cystal induced inflammation in acute gout [11] and in the treatment of other inflammatory diseases of unknown cause [15]. In cardiovascular and metabolic disease, the potential effects of IL1 inhibition are also of interest. Our observations therefore provide a new direction of research into the links between inflammation and hypertension and suggest that IL1 inhibition could be of therapeutic benefit in RVH and CKD.

**Limitation and perspectives:** In the present study, we have not performed time-course inflammatory responses to renal ischemia, which may allow us to observe influx of inflammatory cells, predominantly macrophages, and lymphocytes in the renal arterial clipped kidney in early phase. Also, we have not analyzed the renal function. It is of interest to further confirm the role of IL1β in the development of renin-dependent hypertension using IL-1β -/- mice applied to the 2K1C mouse model.

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