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Protease–activated receptor 2 exacerbates adenine–induced renal tubulointerstitial injury in mice

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ABSTRACT

Hypercoagulability is associated with chronic kidney disease (CKD). Tissue factor/factor VIIa complex and factor Xa in the coagulation cascade are known to activate protease–activated receptor 2 (PAR2), and to cause inflammation and tissue injury. Although PAR2 is highly expressed in the kidney, it is unclear whether PAR2 plays a pathogenic role in CKD. To test this, we fed the mice lacking *Par2* (*F2rl1*$^{-/-}$) and wild type (*F2rl1*$^{+/+}$) mice with adenine diet to induce tubulointerstitial injury, a hallmark of CKD. Adenine-treated mice showed severe renal dysfunction, tubular atrophy, and fibrosis. Fibrin deposition and the expression of tissue factor and PARs markedly increased in their kidneys. Lack of *Par2* attenuated renal histological damage and reduced the expression levels of genes related to inflammation, fibrosis, and oxidative stress. Our data indicate that PAR2 is critically important in the pathogenesis of adenine-induced tubular injury. PAR2 antagonists under development could be useful to treat and prevent CKD.

1. Introduction

CKD increases the risk of cardiovascular complications and death. The number of CKD patients is increasing worldwide [1,2]. Accordingly, there are urgent needs to clarify the pathophysiology of CKD and to identify its therapeutic targets. Several studies indicate that hypercoagulability is associated with CKD. Plasma levels of coagulation parameters are increased with renal dysfunction and inflammation [3,4]. We have clarified that molecules in blood coagulation cascade are of critical importance in the pathogenesis of diabetic nephropathy (DN) [5–7].

Coagulation factors mediate tissue injury through PARs, which consist of four members (PAR1-4). Tissue factor/factor VIIa complex or factor Xa cleaves N terminus of PAR2, activates PAR2, and causes inflammation via NF-kB or MAPK signaling [8]. PAR2 is highly expressed in the kidney, and is suggested to contribute to pathophysiology of specific renal diseases such as DN, crescentic glomerulonephritis and IgA nephropathy in humans or rodents [6,9–12]. However, the importance of PAR2 in the pathogenesis of other common CKD is not clear.

The common pathway for the progression to end stage kidney disease (ESKD) is tubulointerstitial fibrosis and inflammation [13]. Adenine-induced kidney injury is widely used as a model of human CKD [14,15]. The aim of our present study is to clarify whether PAR2 plays a pathological role using this model. We demonstrate that PAR2 is critically important in the progression of tubulointerstitial damage caused by adenine, and suggest that PAR2 probably contributes to the progression of CKD.

2. Materials and methods

2.1. Animals

Male 10-14 week-old wild-type (*F2rl1*$^{+/+}$) and *Par2*$^{-/-}$ (*F2rl1*$^{-/-}$) mice on C57BL/6 J background [16] were fed 0.2% wt/wt adenine-containing diet (Oriental Yeast, Tokyo, Japan) for four weeks to induce tubular injury [14,15]. Mice were individually placed in

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metabolic cages for 24 h before sacrifice. All experiments were conducted in compliance with the guidelines of Tohoku University (2016PhA-039).

2.2. Biochemical measurements in blood and urine

ELISA kits were used to measure plasma cystatin C (R&D Systems, Inc., Minneapolis, MN) and plasma thrombin–antithrombin complex (TAT) (Assaypro, St. Charles, MO). Colorimetric detection kit was used to measure blood urea nitrogen (Arbor Assays, Ann Arbor, MI). Plasma creatinine was determined by the method we developed using LC-MS/MS [17].

2.3. Kidney morphometry

Kidneys were fixed in 2% PFA and embedded in paraffin. The sections 3 μm in thickness were stained with Masson Trichrome or Hematoxylin-Eosin stain to evaluate tubular lesion. Tubulointerstitial damage, defined as total area of tubular atrophic or fibrotic lesion, was traced under blinded manner [18]. For immunohistochemistry, rabbit anti-human fibrin/fibrinogen antibody (1:4000, Dako, Denmark) was used. More than 7 consecutive fields were examined in each slide at 100-fold magnification. The degree of tubular injury or fibrin deposition was examined as the ratio to the entire cortical area. All assessments were performed with Image J (National Institutes of Health, Bethesda, MD).

2.4. Quantitative RT-PCR

Total RNA from the kidney was extracted using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH). *Hypoxanthine-guanine phosphoribosyltransferase* (*Hprt*) was used as a reference gene. All procedure was performed as we previously done [6,19].

2.5. Statistical analyses

Statistical comparisons between two groups were made with the Student's *t*-test for parametric values and Mann-Whitney *U* test for non-parametric values. Multiple groups were compared using two-way ANOVA with the Tukey-Kramer test for parametric values and Kruscal-Wallis test followed by Steel-Dwass test for non-parametric values. All analyses were performed using JMP 11.0.0 (SAS Institute Inc., Cary, NC). Values are presented as mean ± s.e.m. Differences were considered to be statistically significant with $P < 0.05$.

3. Results

3.1. Characteristics of adenine-induced tubulointerstitial injury

Basal characteristics of the mice are shown in Table 1. Adenine diet significantly reduced body weight and renal weight. Adenine deteriorated renal function assessed by plasma creatinine, plasma cystatin C, and blood urea nitrogen. Lack of *Par2* did not ameliorate renal function in our experimental condition.

3.2. Lack of Par2 ameliorates tubular injury and inflammation caused by adenine

Adenine diet induced severe tubular atrophy and lumen dilatation as well as fibrosis in the kidneys (Fig. 1A). Lack of *Par2* significantly reduced area of tubulointerstitial injury (69.8% vs. 77.1% in wild type mice, $P = 0.009$, Fig. 1B). Non-significant recovery of tubular area was observed in *Par2* null mice (31.1% vs. 25.9% in wild type mice, $P = 0.07$, Fig. 1B). Lack of *Par2* significantly reduced the number of white blood cell casts suggesting that lack of *Par2* alleviated inflammation caused by adenine (3.9 vs 1.2/mm^2 in wild type mice, $P = 0.003$, Fig. 1B). Adenine increased renal expression of proinflammatory and profibrotic genes (*Tnfa*, *Tgfb*, *Pai1*, *Fn*, *Acta2* and *Col1*), and lack of *Par2* partially corrected them (Fig. 2A–B). Similarly, lack of *Par2* corrected the levels of macrophage marker *Emr1* elevated by adenine (Fig. 2C).

p47phox, a subunit of NADPH oxidase, produces ROS and increases oxidative stress [20]. Literature shows that the absence of *p47phox* attenuates CKD and DN in rodent models [21,22]. We therefore tested renal expression levels of p47^{phox}, and found that adenine increased it more than 100 fold, and lack of *Par2* partially corrected it (Fig. 2D). These findings indicate that the attenuation of kidney injury in the absence of *Par2* is associated with the amelioration of inflammation and oxidative stress.

3.3. Coagulation and PARs in adenine-induced tubulointerstitial injury

We next characterized changes in coagulation cascade of this model. Although plasma levels of thrombin anti-thrombin complex (TAT) were similar among the groups, adenine significantly increased fibrin deposition and the expression of tissue factor (*Tf*) in the kidney (Fig. 3A–D), suggesting local activation of coagulation in the kidney. Lack of *Par2* did not affect plasma TAT, fibrin deposition, and *Tf* expression (Fig. 3A–D). Adenine significantly elevated levels of *Par1*, *Par2*, and *Par4* expression in the kidneys (Fig. 3E). Interestingly, lack of *Par2* corrected elevated renal expression of both *Par1* and *Par4* in mice given adenine diet. These findings indicate that local activation of coagulation cascade in the kidney, and that increased PARs are likely involved in the pathogenesis of this model.

4. Discussion

In this study we have demonstrated pathological role of PAR2 in adenine-induced tubular injury based on the following findings: Hypercoagulability and increased PAR2 were associated with adenine-induced tubulointerstitial damage (Fig. 3); Lack of *Par2* attenuated histological damage together with gene expression

Table 1
Characteristics of adenine-induced kidney injury in mice.

	Par2$^{+/+}$ (n = 9)	*Par2*$^{-/-}$ (n = 9)	*Par2*$^{+/+}$-Ade (n = 8)	*Par2*$^{-/-}$-Ade (n = 9)
BW (g)	27.4 ± 0.4	25.9 ± 0.7	17.5 ± 0.5^{cd}	19.0 ± 0.7^{cd}
ΔBW (vs. baseline, %)	6.1 ± 1.6	7.2 ± 1.4	−26.1 ± 1.8^{cd}	−28.0 ± 3.8^{cd}
Rt kidney wt (mg)	166.5 ± 5.5	155.1 ± 5.8	132.7 ± 6.5^{ab}	130.2 ± 7.9^{ab}
Rt kidney wt/BW (mg/g)	6.1 ± 0.1	6.0 ± 0.1	7.6 ± 0.4^{ab}	6.8 ± 0.2^{b}
Plasma cystatin C (ng/mL)	317.8 ± 15.3 (8)	423.9 ± 19.7 (8)	1207.3 ± 51.0^{cd}	1122.2 ± 71.7^{cd} (8)
Plasma creatinine (mg/dL)	0.09 ± 0.02 (8)	0.09 ± 0.01 (7)	0.65 ± 0.05^{cd}	0.68 ± 0.06^{cd}
BUN (mg/dL)	26.7 ± 1.7 (8)	24.5 ± 1.0 (8)	146.3 ± 8.7^{cd} (6)	130.6 ± 10.5^{cd} (7)

Data are mean ± s.e.m. Abbreviations: Ade, Adenine; BW, body weight; Wt, weight; BUN, blood urea nitrogen.
Numbers in parentheses indicate numbers of mice studied. [a] $P < 0.05$ vs. WT. [b] $P < 0.05$ vs. *Par2*$^{-/-}$. [c] $P < 0.01$ vs. WT. [d] $P < 0.01$ vs. *Par2*$^{-/-}$.

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