博 士 学 位 論 文

高血圧性糸球体障害における酸化ストレスと トロンボキサン A2 の多様な関連

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Diverse Associations between Oxidative Stress and Thromboxane A2 in Hypertensive Glomerular Injury

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論文題目　Diverse Association between Oxidative Stress and Thromboxane A2 in Hypertensive Glomerular Injury

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Diverse Associations between Oxidative Stress and Thromboxane A_2 in Hypertensive Glomerular Injury

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Abstract

Here we examined the possible contributions of oxidative stress and thromboxane A_2 (TXA_2) to the development of regional heterogeneity in hypertensive glomerular injury using stroke-prone spontaneously hypertensive rats (SHRSP), an animal model of human essential hypertension. We also examined the effect of antioxidant treatment on the regional expression of thromboxane synthase (TXAS) mRNA using a microdissection method. Increases in the glomerular expression of TXAS mRNA were observed in SHRSP at 15 weeks of age compared with that in age-matched normotensive control Wistar-Kyoto (WKY) rats: 2.4-fold and 3.1-fold in superficial and juxtamedullary glomeruli, respectively ($P < 0.05$). Heme oxygenase-1 mRNA expression was markedly increased (>8-fold, $P < 0.05$) in both superficial and juxtamedullary glomeruli in SHRSP compared with the expression in WKY rats. Contrary to our expectations, the treatment of SHRSP with tempol (a superoxide dismutase mimetic) significantly ($P < 0.05$) increased TXAS mRNA expression in superficial glomeruli and improved neither histological injury nor albuminuria, which were aggravated instead. On the other hand, ozagrel (a TXAS inhibitor) had a suppressive effect on TXAS mRNA expression and significantly ($P < 0.05$) improved histological injury. These results indicated that although TXA_2 and oxidative stress are linked to each other, TXA_2 rather than oxidative stress may be a better therapeutic target to improve hypertensive glomerular injury.

Keywords : hypertensive glomerular injury, albuminuria, glomerular hypertension, ozagrel, tempol

Introduction

Despite recent advances in antihypertensive therapy, hypertension is an important risk factor for the progression of chronic kidney disease and can lead to end-stage kidney disease (ESKD) [1, 2]. Thus, in addition to lowering blood pressure, therapeutic strategies to ameliorate hypertensive renal injury need to be established. One possible target is oxidative stress-induced regional ischemia, besed on the results of studies that have reported that hypertensive renal injury, which can be inhibited by tempol (a superoxide dismutase mimetic) [3−5], develops early in the juxtamedullary cortex, the renal region most susceptible renal region to ischemic injuries, and then extends toward more superficial regions with an abundant blood supply [6−8].

In addition to high blood pressure, it is now well recognized that the renin–angiotensin– aldosterone system (RAAS) and vascular inflammation induce renal oxidative stress under the pathophysiology of hypertension [9, 10]. However, there is currently limited understanding of other factors involved in the pathophysiology of hypertension. In the present study, we studied the possible contribution of thromboxane A_2 (TXA_2), a vasopressor eicosanoid that frequently contributes to impaired renal hemodynamics under pathological conditions and potently stimulates reactive oxygen species (ROS) generation, to the development of regional heterogeneity in hypertensive glomerular injury [11, 12]. For this purpose, using an animal model of human essential hypertension, we examined the impact of the inhibition of TXA_2 synthesis in relation to its antioxidant effects on regional glomerular injury. We also determined the regional expression of thromboxane synthase (TXAS) mRNA with and without tempol treatment.

Methods

Animals

Experiments were conducted using male stroke-prone spontaneously hypertensive rats (SHRSP/Kpo) and normotensive control Wistar–Kyoto (WKY/Kpo) rats. These rats were originally established in our laboratories (by Drs Okamoto and Suzuki) [13] and maintained under controlled conditions of temperature (23℃ ± 2℃), humidity (50% ± 10%) and a 12-h light/dark cycle (lights on 07.00−19.00 h) in the Central Research Facilities, Kindai University Faculty of Medicine Center for Animal Experiments. Rats were fed standard rat chow and reverse osmotic water ad libitum. All experimental protocols conformed to the guidelines of the National Institutes of Health (Guide for the Care and Use of Laboratory Animals 1966), and were approved by the Institutional Animal Experimentation Committee of Kindai University Faculty of Medicine (KAME-28-013).

Experimental protocol

Protocol 1. Association between glomerular injury and TXAS and heme oxygenase-1 (HO-1) mRNA expression

Rats were housed in a wire-mesh metabolic cage for 24-h urine collection. Systolic blood pressure (SBP)

of conscious rats at the ages of 5, 10, and 15 weeks was measured using tail-cuff plethysmography (BP-98A; Softron, Tokyo, Japan). Subsequently, rats were anesthetized with an intraperitoneal injection of pentobarbital (~100 mg/kg). Before removing their right kidneys, blood was obtained from the abdominal aorta. Kidneys were perfused with phosphate-buffered saline (PBS) at a perfusion pressure corresponding to the animal's arterial pressure and removed. The removed kidneys were subjected to laser microdissection (LMD) to study mRNA expression or to perform histological analysis.

Protocol 2. Pathological roles of TXA_2 together with oxidative stress

At the age of 10 weeks, SHRSP were divided into three groups: untreated, treated with tempol at 1 mmol/L, or treated with ozagrel (a TXAS inhibitor) at 3.5 mmol/L. As both tempol and ozagrel were readily soluble in water, they were administered by dissolving in the drinking water and provided for 5 weeks. Previous reports found that there was no difference in the blood pressure lowering effect between 1 mmol/L and 3 mmol/L of tempol [14, 15]. We selected tempol at 1 mmol/L since Park et al. [16] showed that this dose of tempol successfully prevented vascular remodeling and progression of hypertension in salt-loaded SHRSP at 16 weeks of age. As regards the dose of ozagrel, Gomi et al. [17] examined the effect of ozagrel with graded doses from 1.4 to 147 mg/kg/day in SHR. They revealed that ozagrel decreases urinary excretion of thromboxane B_2 (TXB_2), which is a stable metabolite of TXA_2, in a dose-dependent manner. SBP and the production of TXB_2 from kidney were decreased only by high dose of ozagrel. Another researcher reported that 100 mg/kg/day of ozagrel improved proteinuria in streptozotocin-induced diabetic rats [18]. Based on these reports, we decided to use ozagrel at 100 mg/kg/day. We converted the dose of 100 mg/kg/day into 3.5 mmol/L of drinking water since the daily water intake of SHRSP maintained in our laboratories is around 30 ml/day. After the 5-week treatment period, SBP and urinary albumin excretion (UAE) were measured, and then the right kidneys were removed using the same method mentioned above.

Measurement of oxidative stress and TXB_2 production

Urine samples were used for measurement of 8-OHdG which is a marker of oxidative stress and TXB_2. Urinary excretion of 8-OHdG was determined using a competitive enzyme linked immunosorbent assay (ELISA) kit (8-OHdG Check; Japan Institute for the Control of Aging, Sizuoka, Japan) [19]. TXB_2 was measured by an enzyme immuno assay (EIA) kit (R&D systems, Minneapolis, MN, USA) following the instructions of the manufacturer [20].

Histological analysis

The removed kidneys were divided into two parts at the center of the minor axis. One part was quickly immersed in OCT compound and immediately frozen in liquid nitrogen for LMD. The other part was fixed with 10% buffered formalin and embedded in paraffin. Subsequently, 4-μm-thick sections were prepared and

stained with periodic acid-Schiff (PAS) to assess renal damage. Regional injury was studied by separately examining the superficial and juxtamedullary glomeruli. Glomerular sclerosis index (GSI) was blindly scored in at least 50 superficial and juxtamedullary glomeruli, in accordance with a previously described method [21]. Each section was scored twice, and the mean score was used for analysis. The degree of sclerosis was scored as follows: 0, no changes; 1, lesions involving <25% of the capillary tuft; 2, lesions affecting 25%－49% of the capillary tuft; 3, lesion involving 50%－75% of the capillary tuft; and 4, lesions involving more >75% of the capillary tuft.

Immunohistochemical analysis

Sections were deparaffinized and the antigenic epitopes were retrieved by microwaving in citrate buffer (10 mmol/L, pH 6.0) for 10 min. Subsequently, to inactivate the endogenous peroxidase activity, the sections were soaked in methanol with 0.3% hydrogen peroxide for 30 min at room temperature to inactivate the endogenous peroxidase activity. After blocking with 5% goat serum for 30 min, sections were incubated overnight at 4℃ with the primary antibodies against an oxidative stress marker 8-hydroxy-2′-deoxyguanosine (8-OHdG, 2.5 μg/ml) (Japan Institute for the Control of Aging, Shizuoka, Japan). The sections were washed in PBS and incubated with biotinylated secondary antibody at 1:300 dilution at room temperature for 30 min and then incubated with avidin–biotin–peroxidase complex (Vectastain ABC kit; Vector Laboratories) for 30 min and visualized with diaminobenzidine tetrahydrochloride.

LMD

LMD was performed using the Leica AS LMD system (Leica Microsystems AG, Wetzlar, Germany), in accordance with the manufacture's manual [22]. Briefly, frozen sections (9－10 μm in thickness) were cut by a cryostat and mounted on a glass slide cover with a 2.5-μm-thick laser pressure-catapulting membrane (PEN foil; Leica Microsystems). The sections were fixed in ethanol:acetic acid (19:1), gently washed with diethylpyrocarbonate (DEPC) -treated water, and stained with 0.05% toluidine blue (TB) solution (pH 7.0, Wako Pure Chemical Industries, Ltd., Osaka, Japan) ; the TB solution was then rinsed with the DEPC-treated water twice, after which the sections were completely air-dried. The superficial and juxtamedullary glomeruli were separately dissected from frozen sections using the LMD system and then immediately collected into a microcentrifuge tube cap filled with lysis buffer.

mRNA level determination

Total RNA from the microdissected glomeruli was extracted using RNeasy mini kit (Qiagen, Valencia, CA). Isolated RNA samples were reverse-transcribed with a high-capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA), and applied to the TaqMan real-time PCR assay (Applied Biosystem) for the following molecules: TXAS (Rn00562160_m1) and HO-1 (Rn00561387_m1). Amplification data were analysed using

Sequence Detection Software (SDS ver. 1.9), and mRNA levels of each target were normalized to the level of β-actin using the $\Delta\Delta$Ct comparative method [23].

Mean glomerular volume (MGV) measurement

MGV was estimated using the 2-profile method [24]. Briefly, kidney specimens were sectioned at 5 μm thickness and stained with PAS. Two sections were made at 20-μm intervals onto sequential slides. At least 10 individual non-sclerotic glomeruli (sclerotic lesions < 75%) were randomly selected from both superficial and juxtamedullary cortices. Glomerular images were obtained using a color video camera attached to a microscope (Nikon Instruments Inc., Melville, NY) at 200 × magnification. In each captured image, the glomerular tuft was digitally traced, and the areas were calculated using computer image analysis software (NIS-Elements D 3.22; Nikon Instruments Inc., Melville, NY). Subsequently MGV was calculated based on the areas of the two sections. We can obtain almost the same results by 2-profile method compared with the Cavalieri method [25], which is the most reliable and accurate method to estimate MGV if more than eight glomeruli are analyzed.

Statistical analysis

All values are expressed as mean ± SEM and were analyzed using unpaired Student's *t*-test and one-way analysis of variance. P values of <0.05 were considered statistically significant.

Results

Association between glomerular injury and TXAS and HO-1 mRNA expression

The characteristics of WKY rats and SHRSP are shown in Table 1. The body weight of SHRSP was significantly ($P < 0.05$) less at the ages of 10 and 15 weeks than that of WKY rats. In SHRSP, SBP increased rapidly as the rats grew and was significantly ($P < 0.05$) high during the entire experimental period compared with that in WKY rats. Although renal function, estimated as serum creatinine concentration (SCr) and creatinine clearance (CCr) /100-g body weight (BW), did not change during the period in WKY rats, it significantly declined in SHRSP at 15 weeks of age. UAE progressively increased only in SHRSP. Urinary TXB_2 excretion was significantly greater in SHRSP than in WKY rats (22.6 ± 2.5 ng/day vs. 11.3 ± 1.0 ng/day, $P < 0.05$). Light microscopy showed high GSI in SHRSP at ≥10 weeks of age. In addition, when we estimated each rat's glomerular injury, much higher GSI was observed in juxtamedullary glomeruli than in superficial glomeruli (Figure 1a). TXAS mRNA expression was enhanced in both superficial and juxtamedullary glomeruli in SHRSP compared with that in age-matched WKY rats (Figure 1b). In superficial glomeruli, this increase almost doubled at 10 weeks of age and remained at this level until 15 weeks of age. On the other hand, in juxtamedullary glomeruli, progressive enhancement occurred and became 3.1-fold at 15 weeks of age. Immunohistochemical analysis revealed greater numbers of 8-OHdG-positive cells in both

glomerulus and tubules in 15-week-old SHRSP than in age-matched WKY rats (Figures 2a and b). Moreover, as expected, urinary 8-OHdG excretion was significantly greater in SHRSP than in WKY rats (830.7 ± 26.6 ng/kg/day vs. 602.3 ± 40.9 ng/kg/day; Figure 2c). HO-1 mRNA expression was also markedly increased in both superficial and juxtamedullary glomeruli (8.7-fold and 11.6-fold, respectively) in SHRSP at 15 weeks of age compared with those in age-matched WKY rats (Figure 2d), although the difference was greater in juxtamedullary glomeruli than in superficial glomeruli.

Table 1. The characteristics of WKY rats and SHRSP at 5, 10, and 15 weeks of age

	WKY rats			SHRSP		
	5 weeks (n = 6)	10 weeks (n = 6)	15 weeks (n = 6)	5 weeks (n = 6)	10 weeks (n = 7)	15 weeks (n = 8)
BW (g)	113 ± 2	321 ± 8	399 ± 5	109 ± 1	266 ± 4*	274 ± 13*
SBP (mmHg)	134.6 ± 4.6	146.7 ± 2.7	140.0 ± 2.0	147.9 ± 2.9*	238.4 ± 6.6*	249.9 ± 5.1*
Serum Cr (mg/dl)	0.20 ± 0.00	0.26 ± 0.00	0.30 ± 0.01	0.16 ± 0.01*	0.24 ± 0.01	0.41 ± 0.03*†
CCr/100-g BW (ml/min/100-g BW)	0.69 ± 0.02	0.71 ± 0.04	0.68 ± 0.04	0.74 ± 0.02	0.74 ± 0.03	0.53 ± 0.06*†
UAE (mg/day)	0.02 ± 0.00	0.12 ± 0.03	0.05 ± 0.01†	0.01 ± 0.00	0.21 ± 0.04*	1.73 ± 0.17*†

Abbreviations: BW, body weight; SBP, systolic blood pressure; Cr, creatinine; CCr, creatinine clearance; UAE, urinary albumin excretion.
Data are indicated as mean ± SEM.
*P < 0.05 vs. WKY rats at the same age.
†P < 0.05 vs. the same species at 10 weeks of age.

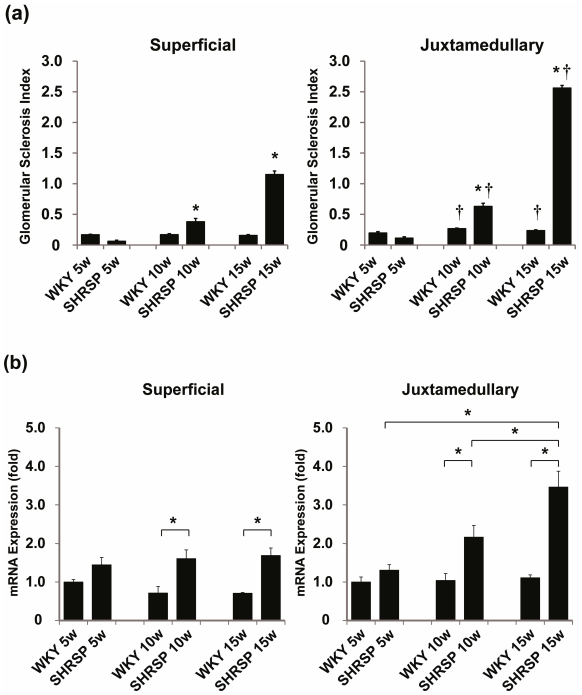

Figure 1. Histological analysis and TXAS mRNA expression in superficial and juxtamedullary glomeruli. (a) GSI of superficial and juxtamedullary glomeruli in WKY rats and SHRSP at 5, 10, and 15 weeks of age. (b) A comparison of TXAS mRNA expression between WKY rats and SHRSP in superficial and juxtamedullary glomeruli. *P < 0.05 vs. WKY rats at the same age, † P < 0.05 vs. superficial glomeruli at the same age.

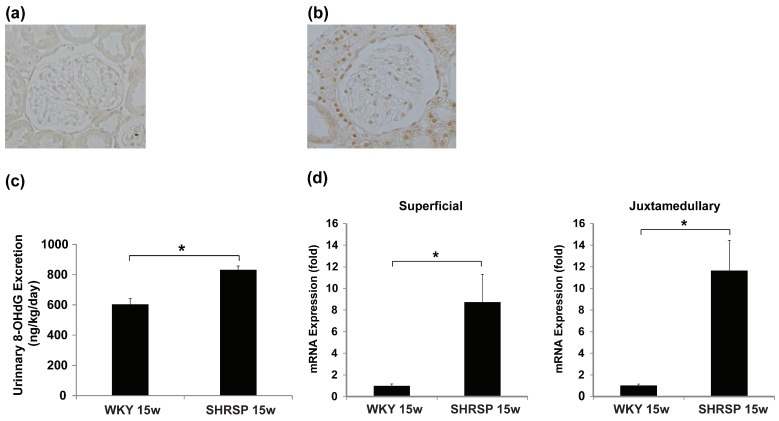

Figure 2. Oxidative stress in the kidneys. (a, b) Immunohistochemical staining for 8-OHdG in WKY rats (a) and SHRSP (b) at 15 weeks of age. Original magnification 100×. (c) Urinary 8-OHdG excretion in WKY rats and SHRSP at 15 weeks of age. (d) A comparison of the HO-1 mRNA expression between WKY rats and SHRSP in superficial and juxtamedullary glomeruli at 15 weeks of age. *P < 0.05.

Pathological roles of TXA_2 together with oxidative stress

Neither tempol nor ozagrel had any effect on BW, SBP, SCr, or CCr/100-g BW in SHRSP (Table 2). On the other hand, both tempol and ozagrel exacerbated UAE (tempol: 17.4 ± 0.2 mg/day, ozagrel: 8.4 ± 1.3 mg/day; Figure 3a), although they reduced urinary 8-OHdG excretion (tempol: 532.3 ± 38.9 ng/kg/day, ozagrel: 616.8 ± 39.2 ng/kg/day; Figure 3b). Ozagrel significantly reduced urinary TXB_2 excretion, but tempol did not have any changes (tempol: 20.0 ± 1.7 ng/day, ozagrel: 12.9 ± 1.3 ng/day; Figure 3c). The exacerbation of UAE was much stronger in tempol-treated rats than in ozagrel-treated ones. In addition, ozagrel, but not tempol improved histological lesions (Figure 4a) and GSI in both superficial and juxtamedullary glomeruli (Figure 4b). Quantitative morphometrical analysis of the glomeruli showed that the MGV of superficial or juxtamedullary glomeruli was 29% or 18% greater in the tempol-treated group, respectively, than in the untreated group. In contrast, no difference was observed in the ozagrel-treated group in either superficial or juxtamedullary glomeruli (Figure 4c). Neither treatment had a significant effect on HO-1 mRNA expression in superficial or juxtamedullary glomeruli (Figure 4d), although both treatments reduced urinary 8-OHdG excretion, as mentioned above (Figure 3b). TXAS mRNA expression is shown in Figure 4e. Tempol significantly increased TXAS mRNA expression in superficial glomeruli (1.4-fold) but not juxtamedullary glomeruli, whereas ozagrel suppressed it. TXAS mRNA expression was significantly less in both superficial and juxtamedullary glomeruli (0.7-fold and 0.7-fold, respectively) in ozagrel-treated rats compared with that in tempol-treated rats.

Table 2. Changes in parameters after treatment

	Untreated (n = 8)	Tempol (n = 8)	Ozagrel (n = 7)
BW (g)	274 ± 13	282 ± 12	262 ± 9
SBP (mmHg)	249.9 ± 5.1	259.2 ± 5.6	252.4 ± 4.9
Serum Cr (mg/dl)	0.41 ± 0.03	0.43 ± 0.2	0.40 ± 0.01
CCr/100-g BW (ml/min/100-g BW)	0.53 ± 0.06	0.51 ± 0.02	0.50 ± 0.01

Abbreviations: BW, body weight; SBP, systolic blood pressure; Cr, creatinine; CCr, creatinine clearance.
Data are indicated as mean ± SEM.
No significant difference was found in any parameters among groups.

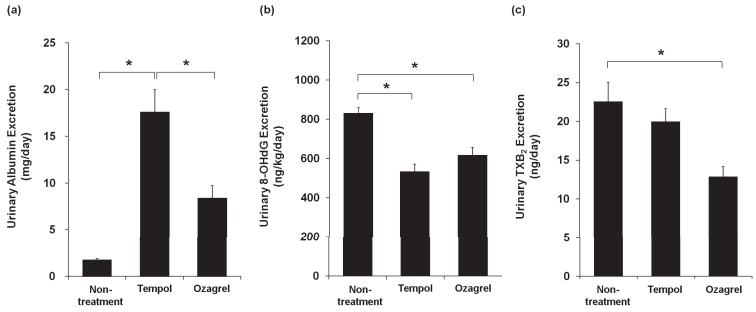

Figure 3. Effect of tempol or ozagrel administration on urinary albumin (a), 8-OHdG (b), and TXB_2 excretion (c). *P < 0.05.

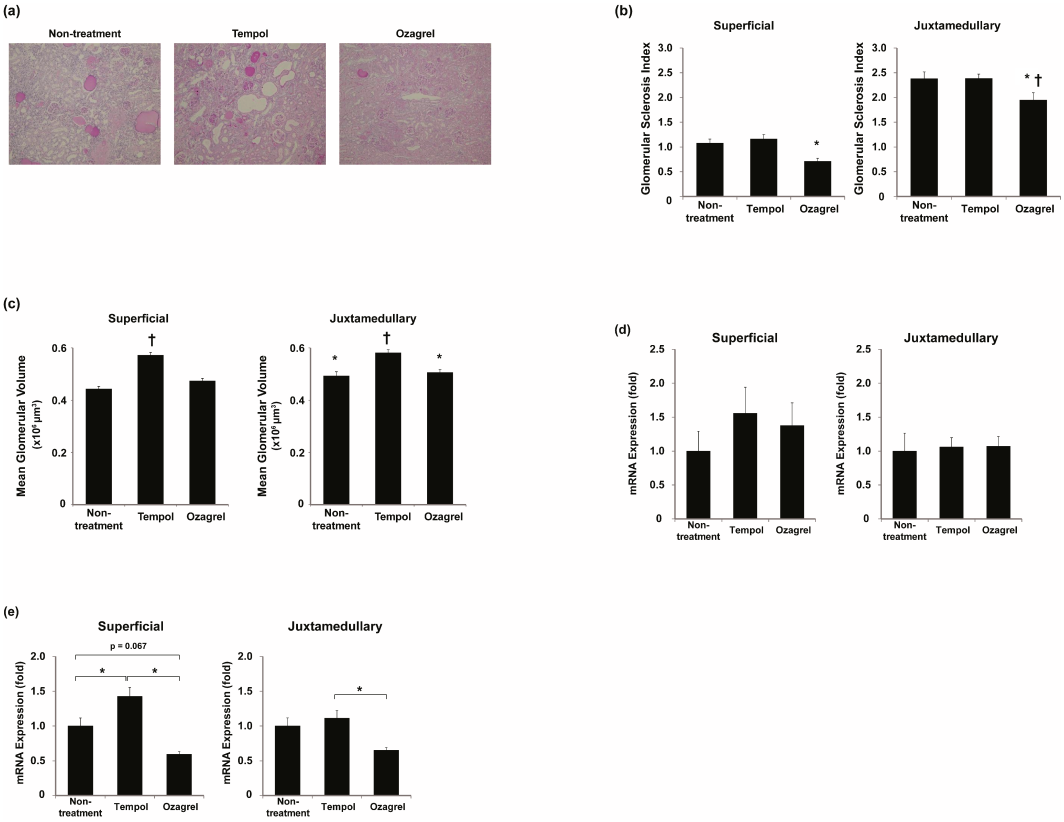

Figure 4. Effect of tempol or ozagrel on histological injury and HO-1 and TXAS mRNA expression. (a) Representative photomicrographs of renal cortex stained with PAS. Original magnification 100×. (b) Glomerular injury estimated by GSI after tempol and ozagrel treatments. Original magnification 100×. *P < 0.05 vs. untreated group, † P < 0.05 vs. tempol-treated group. (c) Morphometrical analysis using the 2-profile method (described in the Methods). *P < 0.05 vs. superficial glomeruli, † P < 0.05 vs. untreated group. (d) (e) Comparisons of HO-1 (d) and TXAS (e) mRNA expression between untreated SHRSP and tempol- or ozagrel-treated SHRSP in superficial and juxtamedullary glomeruli. *P < 0.05.

Discussion

Studies have demonstrated that hypertensive glomerular sclerosis develops early in the juxtamedullary cortex and then extends toward the superficial cortex in rats and humans [6–8]. Although it has been proposed that differences in perfusion pressure or oxidative states between juxtamedullary and superficial glomeruli are responsible for such regional heterogeneity, the precise mechanisms underlying this are unclear. Thus, in the present study, using an animal model of severe essential hypertension, we examined the possible involvements of TXA_2 together with the oxidative stress in the pathophysiology of hypertensive glomerular injury. We confirmed such heterogeneity and indeed found increased oxidative stress together with preferentially enhanced TXAS mRNA expression in the juxtamedullary glomeruli in SHRSP. To our knowledge, this is the first report demonstrating the possible involvement of TXA_2 in regional heterogeneity

in hypertensive glomerular injury. We also found that TXA_2 production can be affected by antioxidant therapy and may affect the degree of UAE.

The importance of oxidative stress in the pathogenesis of hypertensive organ damage has been extensively studied [16, 26–29]. The excessive production or decreased metabolism of ROS, such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl anion (OH^-), can lead to oxidative stress that alters the redox state and causes redirection of redox-regulated signaling pathways and cellular dysfunction or damage [30]. It is now well recognized that RAAS and vascular inflammation induce renal oxidative stress [9, 10]. However, antihypertensive treatment with RAAS blockers may not completely be effective at preventing the progression of hypertensive renal injury to ESKD. Thus, for better antihypertensive treatment, there is a need to determine other factors involved in the pathogenesis of hypertensive glomerular injury. In the present study, we studied the possible involvement of TXA_2 in the pathophysiology of hypertensive glomerular injury as it has been reported that renal synthesis of TXA_2 increase in SHRSP [31]. TXA_2 is an arachidonic acid metabolite converted by TXAS, which by acting on its receptor [thromboxane prostanoid receptor (TPR)] causes platelet aggregation, and contraction of vascular smooth muscle cells and increases the expression of adhesion molecules in endothelial cells [32, 33]. In the kidneys, TXAS and TPR are expressed in endothelial cells and glomerular mesangial cells, and TXA_2 decreases the glomerular filtration rate (GFR) under pathological conditions [34–36]. Gelosa et al. [37] had reported that terutroban, a selective TPR antagonist, prevented vascular hypertrophy and improved the development of proteinuria without affecting blood pressure in SHRSP. This implies that TXA2–TPR pathway mediates glomerular injury through a blood pressure-independent mechanism in severe hypertension. Interestingly, the TXA_2–TPR pathway and oxidative stress form a vicious circle, i.e., TXA_2 stimulates the generation of ROS and O_2^- [11, 12], which in turn stimulates TXA_2 synthesis by TXAS upregulation [38]. This background prompted us to examine their possible associations with the pathogenesis of hypertensive glomerular injury, which we indentified in diverse forms.

We found that both TXAS mRNA expression and oxidative stress increased in SHRSP especially in the juxtamedullary glomeruli, which showed more severe hypertensive injury than that in superficial glomeruli. These findings support the idea that TXA_2 together with oxidative stress contributes to the development of hypertensive glomerular injury. However, tempol showed a tendency to cause marked increases in UAE regardless it significantly reduced renal oxidative stress as estimated by urinary 8-OHdG excretion (Figure 3b), though it may reflect interstitial as well as glomerular oxidative stress. These results are completely consistent with those of Sugama et al. [39], who reported that tempol aggravated renal injury in advanced-stage SHRSP. In addition, some clinical and experimental studies have reported such conflicting results with antioxidant therapies [40, 41]. On the other hand, the increase in UAE was much smaller when TXAS was inhibited with ozagrel, although it reduced renal oxidative stress to an extent similar to that by tempol. Thus, it is possible that antioxidant therapy through TXA_2 inhibition is a better therapeutic target than ROS inhibition to inhibit the aggravation of hypertensive glomerular injury at an advanced stage.

The reason why tempol exacerbated UAE remains unclear. Sugama et al. [39] hypothesized that tempol aggravates renal injury in advanced-stage hypertension by inducing glomerular hypertension in residual nephrons (mostly superficial nephrons) through an inadequate increase in regional blood flow or attenuation of the tubuloglomerular feedback (TGF) response. It has been reported that increased O_2^- production in SHR enhances the TGF response, which is blunted by tempol leading to the development of glomerular hypertension [42]. It has been also demonstrated that increased mechanical stress on glomeruli, such as glomerular hypertension, upregulates TXAS mRNA, resulting in an increased TXA_2 production and further increase in glomerular capillary pressure [43]. These may be the mechanism by which tempol upregulates TXAS mRNA. Consistent with this hypothesis, we found that tempol but not ozagrel increased MGV in both superficial and juxtamedullary cortices (especially superficial cortex) without improving histological lesions and GSI. Thus, tempol may exacerbate UAE by increasing glomerular capillary pressure, whereas ozagrel does not cause glomerular hypertension. In addition to this possibility, TXAS upregulation induced by tempol (especially in superficial glomeruli) may be responsible for the exacerbation of UAE because TXA_2 is known to accelerate glomerular injury through the activation of adhesion molecules or stimulation of mesangial cell proliferation under pathological conditions [32, 44, 45]. However, this possibility is not consistent with our finding that ozagrel significantly (but much less than tempol) increased UAE. Because ozagrel does not increase glomerular capillary pressure nor causes glomerular histological injury (mentioned above), the inhibition of TXA_2 synthesis with ozagrel may increase UAE by the elevation of the glomerular ultrafiltration coefficient under conditions with advanced hypertensive glomerular injury. Consistent with this, TXA_2 is known to decrease the ultrafiltration coefficient and GFR [44]. Alternatively, ozagrel might influence the metabolism of other arachidonates, possibly leading to the exacerbation of UAE. Although the reason ozagrel did not increase CCr/BW is so far unclear, it may be that increased glomerular ultrafiltration coefficient could compensate but not surpass the remained severe glomerular damage. Further studies exploring the precise pathological roles and underlying mechanisms of the involvement of the TXA_2–TPR pathway in the pathophysiology of hypertensive renal injury are clearly required.

Our study has several limitations. First, although we selected the concentration of tempol and ozagrel based on the reason described, the dose-dependent effects of these drugs are to be elucidated. Second, possible involvements of other prostanoids such as prostacyclin in the hypertensive glomerular injury are also to be verified.

In conclusion, the TXA_2–TPR pathway and oxidative stress participate and interact together to promote hypertensive glomerular injury. Our results indicated that antioxidant therapy through TXA_2 inhibition may be a better therapeutic target than ROS inhibition to inhibit the aggravation of hypertensive glomerular injury at an advanced stage.

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Conflict of interest

YN received a scholarship donation from Eli Lilly. SA received honoraria from Takeda, Daiichi-Sankyo, Tanabe-Mitsubishi, Kyowa Hakko Kirin, Novartis, and Taisho-Toyama, as well as scholarship donations from Kyowa Hakko Kirin, Takeda, Daiichi-Sankyo, Tanabe-Mitsubishi, Dainippon-Sumitomo, Torii, and Taisho-Toyama. The remaining authors declare no conflict of interest.

References

1. Siewert-Delle A, Ljungman S, Andersson OK, Wilhelmsen L. Does treated primary hypertension lead to end-stage renal disease? A 20-year follow-up of the Primary Prevention Study in Göteborg, Sweden. Nephrol Dial Transplant 1998;13:3084–3090.
2. Sarnak MJ, Greene T, Wang X, Beck G, Kusek JW, Collins AJ, Levey AS. The effect of a lower target blood pressure on the progression of kidney disease:long-term follow-up of the modification of diet in renal disease study. Ann Intern Med 2005;142:342–351.
3. Hisaki R, Fujita H, Saito F, Kushiro T. Tempol attenuates the development of hypertensive renal injury in Dahl salt-sensitive rats. Am J Hypertens 2005;18:707–713.
4. Nishiyama A, Yao L, Nagai Y, Miyata K, Yoshizumi M, Kagami S, Kondo S, Kiyomoto H, Shokoji T, Kimura S, Kohno M, Abe Y. Possible contributions of reactive oxygen species and mitogen-activated protein kinase to renal injury in aldosterone/salt-induced hypertensive rats. Hypertension 2004;24:841–848.
5. Nishiyama A, Yoshizumi M, Hitomi H, Kagami S, Kondo S, Miyatake A, Fukunaga M, Tamaki T, Kiyomoto H, Kohno M, Shokoji T, Kimura S, Abe Y. The SOD mimetic tempol ameliorates glomerular injury and reduces mitogen-activated protein kinase activity in Dahl salt-sensitive rats. J Am Soc Nephrol 2004;15:306–315.
6. Bohle A, Biwer E, Christensen JA. Hyperperfusion injury of the human kidney in different glomerular diseases. Am J Nephrol 1988;8:179–186.
7. Iversen BM, Amann K, Kvam FI, Wang X, Ofstad J. Increased glomerular capillary pressure and size mediate glomerulosclerosis in SHR juxtamedullary cortex. Am J Physiol 1998;274:F365–F373.
8. Mori T, Cowley AW Jr. Role of pressure in angiotensin II-induced renal injury:chronic servo-control of renal perfusion pressure in rats. Hypertension 2004;43:752–759.
9. Higuchi S, Ohtsu H, Suzuki H, Shirai H, Frank GD, Eguchi S. Angiotensin II signal transduction through the AT1 receptor:novel insights into mechanisms and pathophysiology. Clin Sci (Lond) 2007;112:417–428.
10. Yao EH, Fukuda N, Matsumoto T, Kobayashi N, Katakawa M, Yamamoto C, Tsunemi A, Suzuki R, Ueno T, Matsumoto K. Losartan improves the impaired function of endothelial progenitor cells in hypertension via an antioxidant effect. Hypertens Res 2007;30:1119–1128.
11. Wang C, Luo Z, Kohan D, Wellstein A, Jose PA, Welch WJ, Wilcox CS, Wang D. Thromboxane prostanoid receptors enhance contractions, endothelin-1 and oxidative stress in microvessels from mice with CKD. Hypertension 2015;65:1055–1063.
12. Ellinsworth DC, Shukla N, Fleming I, Jeremy JY. Interactions between thromboxane A_2, thromboxane/prostaglandin (TP) receptors, and endothelium-derived hyperpolarization. Cardiovasc Res 2014;102:9–16.

13. Okamoto K, Yamamoto K, Morita N, Ohta Y, Chikugo T, Higashizawa T, Suzuki T. Establishment and use of the M strain of stroke-prone spontaneously hypertensive rat. J Hypertens 1986;4:S21–S24.
14. Simonsen U, Christensen FH, Buus NH. The effect of tempol on endothelium-dependent vasodilatation and blood pressure. Pharmacol Ther. 2009;122:109–124.
15. Dornas WC, Silva M, Tavares R, de Lima WG, dos Santos RC, Pedrosa ML, Silva ME. Efficacy of the superoxide dismutase mimetic tempol in animal hypertension models:a meta-analysis. J Hypertens. 2015;33:14–23.
16. Park, JB, Touyz, RM, Chen, X, Schiffrin EL. Chronic treatment with a superoxide dismutase mimetic prevents vascular remodeling and progression of hypertension in salt-loaded stroke-prone spontaneously hypertensive rats. Am J Hypertens 2002;15:78－84.
17. Gomi T, Ikeda T, Ishimitsu T, Uehara Y. Protective effect of thromboxane synthetase inhibitor on hypertensive renal damage in Dahl salt-sensitive rats. Prostaglandins Leukot Essent Fatty Acids 1989;37:139–144.
18. Hora K, Oguchi H, Furukawa T, Hora K, Tokunaga S. Effects of a selective thromboxane synthetase inhibitor OKY-046 on experimental diabetic nephropathy. Nephron. 1990;56:297–305.
19. Negishi H, Njelekela M, Ikeda K, Sagara M, Noguchi T, Kuga S, Kanda T, Liu L, Nara Y, Tagami M, Yamori Y. Assessment of in vivo oxidative stress in hypertensive rats and hypertensive subjects in Tanzania, Africa. Hypertens Res. 2000;23:285–289.
20. Pidgeon GP, Tamosiuniene R, Chen G, Leonard I, Belton O, Bradford A, Fitzgerald DJ. Intravascular thrombosis after hypoxia-induced pulmonary hypertension:regulation by cyclooxygenase-2. Circulation. 2004;110:2701–2707.
21. Raij L, Azar S, Keane W. Mesangial immune injury, hypertension, and progressive glomerular damage in Dahl rats. Kidney Int 1984;26:137–143.
22. Kobayashi T, Uehara S, Ikeda T, Itadani H, Kotani H. Vitamin D_3 up-regulated protein-1 regulates collagen expression in mesangial cells. Kidney Int 2003;64:1632–1642.
23. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta C(T)) Method. Methods 2001;25:402–408.
24. Najafian B, Basgen JM, Mauer M. Estimating mean glomerular volume using two arbitrary parallel sections. J Am Soc Nephrol 2002;13:2697–2705.
25. Gundersen HJ, Jensen EB. The efficiency of systematic sampling in stereology and its prediction. J Microsc 1987;147:229–263.
26. Niazi ZR, Silva GC, Ribeiro TP, León-González AJ, Kassem M, Mirajkar A, Alvi A, Abbas M, Zgheel F, Schini-Kerth VB, Auger C. EPA:DHA 6:1 prevents angiotensin II-induced hypertension and endothelial dysfunction in rats:role of NADPH oxidase- and COX-derived oxidative stress. Hypertens Res 2017;40:966–975.

27. Kitiyakara C, Chabrashvili T, Chen Y, Blau J, Karber A, Aslam S, WelchWJ, Wilcox CS. Salt intake, oxidative stress, and renal expression of NADPH oxidase and superoxide dismutase. J Am Soc Nephrol 2003;14:2775–2782.
28. Tanito M, Nakamura H, Kwon YW, Teratani A, Masutani H, Shioji K, Kishimoto C, Ohira A, Horie R, Yodoi J. Enhanced oxidative stress and impaired thioredoxin expression in spontaneously hypertensive rats. Antioxid Redox Signal 2004;6:89–97.
29. Wilcox CS. Oxidative stress and nitric oxide deficiency in the kidney:a critical link to hypertension? Am J Physiol 2005;289:R913–R935.
30. Wilcox CS. Reactive oxygen species:roles in blood pressure and kidney function. Curr Hypertens Rep 2002;4:160–166.
31. Kawaguchi H, Saito H, Yasuda H:Renal prostaglandins and phospholipase A_2 in spontaneously hypertensive rats. J Hypertens 1987;5:299–304.
32. Ishizuka T, Suzuki K, Kawakami M, Hidaka T, Matsuki Y, Nakamura H. Thromboxane A_2 receptor blockade suppresses intercellular adhesion molecule-1 expression by stimulated vascular endothelial cells. Eur J Pharmacol 1996;312:367–377.
33. Davì G, Patrono C. Platelet activation and atherothrombosis. N Engl J Med 2007;357:2482–2494.
34. Spurney RF, Fan PY, Ruiz P, Sanfilippo F, Pisetsky DS, Coffman TM. Thromboxane receptor blockade reduces renal injury in murine lupus nephritis. Kidney Int 1992;41:973–982.
35. Abe T, Takeuchi K, Takahashi N, Tsutsumi E, Taniyama Y, Abe K. Rat kidney thromboxane receptor:molecular cloning, signal transduction and intrarenal expression localization. J Clin Invest 1995;96:657–664.
36. Kobayashi T, Suzuki J, Watanabe M, Suzuki S, Yoshida K, Kume K, Suzuki H. Changes in platelet calcium concentration by thromboxane A_2 stimulation in patients with nephrotic syndrome of childhood. Nephron 1997;77:309–314.
37. Gelosa P, Sevin G, Pignieri A, Budelli S, Castiglioni L, Blanc-Guillemaud V, Lerond L, Tremoli E, Sironi L. Terutroban, a thromboxane/prostaglandin endoperoxide receptor antagonist, prevents hypertensive vascular hypertrophy and fibrosis. Am J Physiol Heart Circ Physiol. 2011;300:H762–768.
38. Korbecki J, Baranowska Bosiacka I, Gutowska I, Chlubek D:The effect of reactive oxygen species on the synthesis of prostanoids from arachidonic acid. J Physiol Pharmacol 2013;64:409–421.
39. Sugama I, Kohagura K, Yamazato M, Nakamura T, Shinzato T, Ohya Y. Superoxide dismutase mimetic, tempol, aggravates renal injury in advanced-stage stroke-prone spontaneously hypertensive rats. J Hypertens 2014;32:534–541.
40. Jialal I, Devaraj S. Antioxidants and atherosclerosis:don't throw out the baby with the bath water. Circulation 2003;107:926–928.

41. Bjelakovic G, Nikolova D, Gluud LL, Simonetti RG, Gluud C. Antioxidant supplements for prevention of mortality in healthy participants and patients with various diseases. Cochrane Database Syst Rev 2012;3:CD007176.
42. Wilcox CS. Redox regulation of the afferent arteriole and tubuloglomerular feedback. Acta Physiol Scand 2003;179:217–223.
43. Anwar MA Shalhoub J Lim CS Gohel MS Davies AH. The Effect of Pressure-Induced Mechanical Stretch on Vascular Wall Differential Gene Expression J Vasc Res 2012;49:463–478.
44. Remuzzi G, FitzGerald GA, Patrono C. Thromboxane synthesis and action within the kidney. Kidney Int 1992;41:1483–1493.
45. Spurney RF, Onorato JJ, Albers FJ, Coffman TM. Thromboxane binding and signal transduction in rat glomerular mesangial cells. Am J Physiol 1993;264:F292–F299.