Renal denervation reduces glomerular injury by suppressing NAD(P)H oxidase activity in Dahl salt-sensitive rats

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Abstract

Background. Renal sympathetic nerve activity has important effects on renal function in chronic kidney disease. Recent studies indicated that beta agonists directly stimulate NAD(P)H oxidase in endothelial cells. Therefore, we investigated whether renal denervation protects renal function through an anti-oxidative effect.

Methods. The right kidney was removed from Dahl salt-sensitive hypertensive rats. Two weeks later, the rats underwent either left renal denervation (Nx-RDNx; n = 10) or a sham operation (Nx-Sham; n = 10). After a further 6 weeks, kidney function and renal tissue were assessed.

Results. Renal denervation reduced glomerular injury by suppressing NAD(P)H oxidase activity in Dahl salt-sensitive rats.

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Results. After the Nx-RDNx or Nx-Sham surgery, urinary albumin excretion and the histologic glomerular sclerosis index were lower in the Nx-RDNx group than in the Nx-Sham group. Fluorescence staining for reactive oxygen species in isolated glomeruli was significantly weaker in the Nx-RDNx group. A lucigenin assay of NAD(P)H oxidase activity in isolated glomeruli indicated that renal denervation may have caused the reduction in reactive oxygen species through suppression of the activity of NAD(P)H oxidase. The levels of mRNA for NAD(P)H oxidase components and the levels of rac1 were higher in glomeruli from the Nx-Sham group than from the Nx-RDNx group. In this ex vivo study, although the NAD(P)H oxidase activity did not change with administration of either the α- or β2-agonist, it increased with the β1-agonist.

Conclusions. Renal sympathetic denervation helps to protect against glomerular sclerosis, possibly by suppressing NAD(P)H oxidase activity, thereby decreasing glomerular reactive oxygen species.

Keywords: chronic kidney disease; NAD(P)H oxidase; rac1; reactive oxygen species; sympathetic nerve

Introduction

Chronic kidney disease (CKD) is a risk factor for the composite outcome of all-cause mortality and cardiovascular disease in the general population [1, 2], and the importance of inhibiting the progression of CKD is now recognized. Age, obesity, diabetes, hypertension and smoking are all predictors of the development of new-onset kidney disease [3]. In addition, activation of the renin–angiotensin system (RAS), insulin resistance and sympathetic nerve activity may all have a role [4, 5]. Sympathetic nerve activity (SNA) is increased in CKD patients [6, 7] and plays an important and distinct role in renal disease-associated hyperperfusion. Indeed, sympathetic activation is of crucial importance for both the raised blood pressure and the high morbidity and mortality of end-stage renal disease [8].

The renal vasculature is richly supplied by sympathetic nerves [9], and renal SNA significantly affects pre- and post-glomerular vascular resistance [10, 11]. Thus, renal function is closely linked with SNA. The Glycemic Effect in Diabetes Mellitus: Carvedilol–Metoprolol Comparison in Hypertensives (GEMINI) trial is the first randomized, double-blind, controlled clinical trial to evaluate the effects of adding β-blockers to standard therapy with RAS blockade to achieve blood pressure control in patients with diabetes and hypertension [12]. In this trial, carvedilol has been shown to decrease urinary albumin excretion and protect renal function. This suggests that renal sympathetic hyperactivity may play a role in progressive renal dysfunction. However, the mechanism of renoprotection via sympathetic nerve suppression remains unclear.

We have previously shown that reactive oxygen species (ROS) contribute to the initiation and development of glomerular injury, and reduction of oxidative stress is effective for ameliorating renal tissue injury [13–15]. ROS and oxidative stress are involved in tissue damage and the onset, development, and maintenance of vascular lesions induced by hypertension, diabetes, obesity, and aging. Moreover, increased levels of ROS activate the RAS and sympathetic nervous system [16]. Thus, oxidative stress may be involved in the mechanism of renal damage as a result of sympathetic nerve activation. Therefore, we hypothesized that activation of the sympathetic nervous system increases oxidative stress in the glomeruli and leads to progression of renal damage. We undertook the present study to investigate whether renal denervation reduces ROS in glomeruli and improves renal damage in Dahl salt-sensitive hypertensive rats.

Materials and methods

Experimental protocol and tissue preparation

The experimental protocol (No. 08-068) was approved in advance by the Ethics Review Committee for Animal Experimentation of the Kawasaki Medical School, Kurashiki, Japan. Male Dahl salt-sensitive rats (160 to 180 g) were purchased from Charles River Japan (Kanagawa, Japan). The animals were housed in a temperature- and humidity-controlled room with a 12:12-h light–dark cycle (light on 7:00–19:00); they were fed standard laboratory animal chow and had free access to tap water. The rats were randomly divided into four groups. The animals in two groups had the right kidney removed (uninephrectomy; Nx) under anesthesia, and those in the other two groups had sham surgery (DL group or DH group; n = 10 in each group). Two weeks later, half of the Nx group underwent left renal denervation (Nx-RDNx group; n = 10), and the other half had sham surgery (Nx-Sham; n = 10). The DL group received a 0.3% salt diet, and the DH, Nx-RDNx and Nx-Sham groups received an 8% diet after renal denervation (RDNx) or sham operation. RDNx was performed as described previously [17]. Following abdominal midline incision, the left kidney was exposed and the renal arteries and veins isolated from the surrounding connective tissue. After stripping the visible nerves, the vessels were painted for 2 min with a solution of 10% phenol in absolute ethyl alcohol. The animals recovered from anesthesia 10–20 min after the end of surgery. In the sham operation, the renal nerves were isolated but preserved. After stripping the visible nerves, the vessels were painted for 2 min with phosphate-buffered saline (PBS). It has been demonstrated that the presence of phenol-containing solution reduces renal catecholamine levels and, specifically, renal norepinephrine (NA) concentration to <5% of control [18].

During the experimental period, body weight was measured weekly. Six weeks after the denervation or sham operation, the rats were subjected to a 24-h fast in metabolic cages to collect urine samples. Urinary albumin concentrations were measured spectrophotometrically using the Nephat enzyme-linked immunosorbent assay kit (Exocell, Philadelphia, PA, USA). Urinary creatinine levels and sodium concentrations were also measured. The rats were sacrificed, and blood samples were obtained with an 18-gauge needle inserted into the left ventricle [19]. The abdominal aorta was cannulated, and the kidney was perfused in a retrograde manner with ice-cold PBS (pH 7.4). Serum creatinine levels were also measured for calculation of creatinine clearance. One-quarter of the removed kidneys were immersed and fixed in 4% paraformaldehyde and then embedded in paraffin for periodic acid–Schiff staining, and another one-quarter of the kidneys were frozen fixed for immunohistochemistry. A small amount of the kidneys was immediately frozen and used for NA determination. The rest (approximately half) of the removed kidneys from each rat were cut into small pieces, and the glomeruli were isolated for protein and RNA extraction, superoxide measurement and NAD(P)H oxidase activity assay using the mechanical graded sieving technique [13]. After isolation, the purity of the final suspension was determined by light microscopy. On average, there was 5% tubular contamination.

Blood pressure and heart rate measurement

The rats underwent implantation of a telemetry system to monitor blood pressure and heart rate. The system consists of a radiofrequency transmitter (TA11PAC40, Data Sciences, Minneapolis, MN, USA), a receiver panel (model RA1010, Data Sciences) and an acquisition system (Dataquest 4 system, Data Sciences). The catheter of the transducer was implanted into
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the abdominal aorta just below the bifurcation of the renal arteries, and the sensor itself was fixed to the peritoneum. After implantation, the rats were allowed to recover from the operation for 38 to 40 days; at this time, the telemetry tracing indicated re-establishment of the 24-h oscillations of blood pressure and heart rate.

Measurement of kidney norepinephrine concentration

Kidney tissue was sonicated in 0.5 mL of buffer (pH 4.0) containing 0.17 mol/L citrate–acetate and 10% methanol. The sonicated mixture was centrifuged, and the clear aspirate was subjected to microfiltration before measurement of NA concentration using an enzyme-linked immunoabsorbent assay.

Histopathologic examination

Paraffin-embedded kidney sections approximately 4 μm thick were deparaffinized and stained with periodic acid–Schiff. In each group, the percent age of those exhibiting segmental sclerosis in a total of over 200 glomeruli was determined and expressed as glomerular sclerosis index. Glomerular sclerosis was graded semiquantitatively in each glomerulus using a scale of 0 to 3: 0, no glomerular sclerosis; 1, segmental glomerular sclerosis in <25% of glomeruli; 2, segmental glomerular sclerosis in 25% to 50% of glomeruli; and 3, segmental glomerular sclerosis in >50% of glomeruli. The mean score per glomerulus was determined for each rat and reported as the glomerular sclerosis index [15].

Immunohistochemical study

Cryostat kidney sections approximately 4 μm thick were used for immunohistochemical analysis of neuropeptide Y (NPY), which is a sympathet ic neurotransmitter. A goat anti-NPY monoclonal antibody (sc-14727; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the primary antibody. Fluorescein isothiocyanate-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology) was used for the secondary antibody. Paraffin-embedded kidney sections approximately 4 μm thick were deparaff inized. A mouse anti-desmin monoclonal antibody (Dako Japan, Tokyo, Japan) was used as the primary antibody. Antibody binding was detected using a Histofine Simple Stain MAX-PO (MULTI) kit (Nichirei, Tokyo, Japan) and 3,3'-diaminobenzidine (Sigma-Aldrich Japan). In the case of immunostaining for desmin, the outer cell layer of the glomerular tuft was evaluated to determine the desmin score, as described previously [20]; 0, from 0% to 5% staining; 1, from 5% to 25%; 2, from 25% to 50%; 3, from 50% to 75%; 4, greater than 75%. At least 20 glomeruli were randomly selected in each rat (n = 10), and the mean score was calculated (total over 200 glomeruli). The percentage of staining in the glomeruli was measured using WinROOF software (MITANI Co., Japan).

Detection of superoxide in glomeruli

Superoxide production was detected by 2′,7′-dichlorofluorescein (DCF) staining [20]. Isolated glomeruli from each group were incubated with RPMI-1640 containing 20 mol/L 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Molecular Probes, Eugene, OR, USA) for 10 min and then rinsed with PBS. Fluorescence images were obtained using a confocal laser microscope (Leica Microsystems, Tokyo, Japan) at excitation/emission wavelengths of 485/535 nm for DCF. The fluorescence intensity values from 20 different isolated glomeruli were calculated by Leica TCS-NT software (Leica Microsystems), and the average values are presented.

Lucigenin chemiluminescence assay for measurement of NAD(P)H oxidase activity in isolated glomeruli

NAD(P)H oxidase activity in the glomeruli was measured using lucigenin chemiluminescence as previously described [21]. Lucigenin chemiluminescence was expressed in units per minute per milligram.

RNA isolation and real-time quantitative PCR

Total RNA was isolated from the glomeruli with TRIzol (Invitrogen Japan, Tokyo, Japan). Reverse transcriptase reactions were performed using a Ready-To-Go T-Primed First-Strand kit (GE Healthcare Bio-Sciences, Tokyo, Japan) for first-strand cDNA synthesis. Real-time quantitative PCR was performed using the ABI Prism 7700 sequence-detection system (Applied Biosystems, Foster City, CA, USA). Primers and probes for TaqMan analysis were designed using Primer Express 1.5 (Applied Biosystems) with information from the supplier based on the sequence information from GenBank or EST databases. The primers and probes used for angiotensin 1 receptor (AT1R), angiotensin-converting enzyme (ACE), p22phox, p47phox and p67phox (where phox indicates phagocyte oxidase) were described in our previous study [22]. The primers and probe for rat gp91phox (NM_023965) were as follows: 5′-AAGGAGTGCAGCAGAAAGT-3′ (forward primer); 5′-TACAGGAACATGGGACCCACGAT-3′ (reverse primer); and 5′-FAM-CGGGAAAACCTTCTTATGACTTGGAAAATG-TAMRA-3′ (TaqMan probe).

Western blot analysis

Proteins extracted from the isolated glomeruli were used for rat immunoblotting. Extracted proteins (10 μg) were separated in a 15% sodium dodecyl sulfate-polyacrylamide gel and then transferred onto a polyvinyldene difluoride membrane. The membranes were incubated for 1 h with anti-rac1 (Abcam Japan, Tokyo, Japan) and anti-actin antibodies (Santa Cruz Biotechnology). Next, the blots were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology). Bands were visualized using the ECL Western blotting detection system (GE Healthcare Bio-Sciences). The integrated density (density 3 area) of the bands was quantified using Image-J software (http://rsbweb.nih.gov/ij/).

Ex vivo NAD(P)H oxidase activity in glomeruli exposed to catecholamine agonists

Glomeruli were isolated from 8-week-old Sprague-Dawley (SD) rats (n = 10) and incubated for 30 min with one of the following four substances: PBS (control), dobutamine (10−7 M; a selective β1-adrenergic receptor agonist), salbutamol (10−6 M; a selective β2-adrenergic receptor agonist) and phenylephrine (10−6 M; a selective α-adrenergic receptor agonist). After incubating the glomeruli for 30 min, we measured the NAD (P)H oxidase activity using a lucigenin-enhanced chemiluminescence assay. We also tested different doses of dobutamine (10−7, 10−6, 10−5 M) with or without atenolol (10−7 M; β1-adrenoceptor antagonists) to determine whether there was a dose-dependent effect on the isolated glomeruli.

Statistical analysis

Values are expressed as mean ± standard error of the mean (SEM). Statistical comparisons were made using the Mann–Whitney U test or the one-factor analysis of variance with a Tukey–Kramer test for multiple comparisons. A P-value <0.05 was considered significant.

Results

Renal neuropeptide Y staining and tissue catecholamines

Renal innervation of experimental rats was analysed in cryostat sections stained for NPY to identify sympathetic nerve fibers. Figure 1A shows that NPY was detected in the DH and Nx-Sham groups but not in the Nx-RDNx group. The renal tissue NA concentration was reduced in the Nx-RDNx group compared with the Nx-Sham group (Figure 1B).

Physiologic and biochemical parameters

Table 1 summarizes the physiologic and biochemical parameters in each group. Kidney weight and urinary albumin excretion were significantly increased, and creatinine clearance was significantly decreased by uninephrectomy (P < 0.05 vs DH). Systolic blood pressure, heart rate, body weight and urinary sodium excretion were not changed af-
ter uninephrectomy. Six weeks after the denervation of sham surgeries, systolic blood pressure, heart rate, body weight, kidney weight and urinary sodium excretion did not differ between Nx-Sham and Nx-RDNx groups. Creatinine clearance was significantly higher, and urinary albumin excretion was significantly lower in the Nx-RDNx group than in the Nx-Sham group (P < 0.05). This suggests that RDNx protects renal function. Figure 2 shows the trend of systolic blood pressure every 2 weeks after RDNx and for 24 h at 6 weeks after RDNx. There was no significant difference in systolic blood pressure between the Nx-Sham and Nx-RDNx groups in either 24-h or long-term blood pressure changes.

Morphologic study

Figure 3A shows a representative histology of the glomeruli. The glomerular sclerosis score (Figure 3B) was increased in the Nx-Sham compared with the DH group (glomerular sclerosis score: DH, 1.6 ± 0.5; Nx-Sham, 2.4 ± 0.3; P < 0.05), and the score was significantly lower in the Nx-RDNx group than in the Nx-Sham group (glomerular sclerosis score: Nx-RDNx, 1.3 ± 0.4; P < 0.05 vs Nx-Sham). This suggests that RDNx prevented glomerular damage. Figure 3C and D show desmin staining and the score as podocyte injury marker. Desmin staining was much higher in the Nx-Sham group than in the DH group (desmin score: DH, 1.9 ± 0.3; Nx-Sham, 3.2 ± 0.2; P < 0.05), and lower in the Nx-RDNx group than in the Nx-Sham group (Nx-RDNx, 2.2 ± 0.2; P < 0.05 vs Nx-Sham). These data support the hypothesis that RDNx prevented glomerular podocyte damage.

Glomerular ROS production

Oxidation of DCFH to the fluorescent compound DCF was used as a qualitative marker of cellular oxidative stress because a number of chemicals, such as H$_2$O$_2$, ONOO$^-$ and HOCl, can cause DCF fluorescence [23]. Figure 4A shows representative glomerular fluorescence images in each group. The DCF fluorescence intensity in the isolated glomeruli was significantly stronger in the Nx-Sham group than in the DH group (2.5 ± 0.2-fold, P < 0.05 vs DH) and weaker in the Nx-RDNx group than in the Nx-Sham group (1.8 ± 0.3-fold, P < 0.05 vs Nx-Sham). We determined NAD(P)H oxidase activity in isolated glomeruli with a lucigenin chemiluminescence assay (Figure 4B). ROS production by NAD(P)H oxidase was significantly lower in the Nx-RDNx group than in the Nx-Sham group (212 ± 12 vs 422 ± 19 RLU/min/mg tissue, respectively, P < 0.05). These results suggest that RDNx decreased superoxide production by reduction of NAD(P)H oxidase activation.

Glomerular mRNA expression for NAD(P)H oxidase components

p22phox and gp91phox are membrane components, whereas p47phox and p67phox are cytosolic components of NAD(P)H oxidase. Expression of mRNAs of both cytosolic components (Figure 5A and B) was significantly increased by uninephrectomy (p47phox: 1.6 ± 0.1-fold, P < 0.05 vs DH; p67phox: 1.7 ± 0.1-fold, P < 0.05 vs DH), and the expression was decreased by RDNx (p47phox: 1.2 ± 0.1-fold, P < 0.05 vs Nx-Sham; p67phox: 1.3 ± 0.1-fold, P < 0.05 vs DH).
By contrast, expression of mRNAs of the membrane components did not significantly differ between the groups (Figure 5C and D).

**Glomerular expression of rac1 protein**

A previous study has suggested that Rho-like small GTPase rac1 is important in the activation of NAD(P)H oxidase [24]. We determined the expression of total glomerular rac1 protein (Figure 6) by Western blot analysis. The rac1 protein expression was significantly increased by uninephrectomy (1.5 ± 0.2-fold, \( P < 0.05 \) vs DH) and significantly lower in glomeruli from the Nx-RDNx group than from the Nx-Sham group (1.1 ± 0.2-fold, \( P < 0.05 \) vs Nx-Sham).

**Renin–angiotensin system in glomeruli and systemic renin activity**

We examined the contribution of the renin–angiotensin system (RAS) in this study. AT1R mRNA expression was not significantly different in each group (Figure 7A). ACE mRNA expression in isolated glomeruli was significantly increased in both the Nx-Sham and Nx-RDNx groups compared with the non-nephrectomized DH group (2.4 ± 0.3-fold and 2.3 ± 0.4-fold, respectively, \( P < 0.05 \) vs DH). However, there was no significant difference between the Nx-Sham and Nx-RDNx groups (Figure 7B). Plasma renin activity (Figure 7C) was significantly increased in the Nx-Sham group compared with DH control group (Nx-Sham, 1.4 ± 0.3 ng/mL/min; DH, 0.5 ± 0.2 ng/mL/min; \( P < 0.05 \)), and RDNx suppressed this activity (Nx-RDNx, 0.7 ± 0.2 ng/mL/min, \( P < 0.05 \) vs Nx-Sham).

**Ex vivo catecholamine-stimulated NAD(P)H oxidase activity in isolated glomeruli**

Using isolated glomeruli, we investigated the direct effects of catecholamines on NAD(P)H oxidase activity. Although NAD(P)H oxidase activity did not change with the administration of either the \( \alpha \)-(phenylephrine) or \( \beta_2 \)-agonist (salbutamol), it increased with the administration of the \( \beta_1 \)-agonist (dobutamine) (Figure 8A). Furthermore, an increase in the dose of the \( \beta_1 \)-agonist led to an increase in NAD(P)H oxidase activity, and the activation was blocked by the \( \beta_1 \)-antagonist (atenolol) (Figure 8B).

**Discussion**

In the present study, we explored whether RDNx in rats with sympathetic hyperactivity reduces oxidative stress in the glomeruli, independent of lowering blood pressure. In

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**Table 1. Physiologic data and biochemical data**

<table>
<thead>
<tr>
<th>Condition</th>
<th>BW (g)</th>
<th>SBP (mmHg)</th>
<th>HR (min)</th>
<th>KW (mg/g·BW)</th>
<th>U-Alb (mg/g·CRN)</th>
<th>U-Na (mg/day)</th>
<th>Ccr (mL/min/100 g·BW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL (n = 10)</td>
<td>334 ± 17</td>
<td>105 ± 8</td>
<td>401 ± 25</td>
<td>4.7 ± 0.3</td>
<td>5.0 ± 0.5</td>
<td>0.5 ± 0.1</td>
<td>1.46 ± 0.11</td>
</tr>
<tr>
<td>DH (n = 10)</td>
<td>352 ± 14</td>
<td>176 ± 11</td>
<td>465 ± 18</td>
<td>5.1 ± 0.2</td>
<td>40.3 ± 4.5</td>
<td>2.7 ± 0.4</td>
<td>1.05 ± 0.11</td>
</tr>
<tr>
<td>Nx-Sham (n = 10)</td>
<td>350 ± 19</td>
<td>185 ± 8</td>
<td>459 ± 22</td>
<td>7.9 ± 0.3 *</td>
<td>75.3 ± 5.5 *</td>
<td>2.3 ± 0.3</td>
<td>0.62 ± 0.05 *</td>
</tr>
<tr>
<td>Nx-RDNx (n = 10)</td>
<td>347 ± 12</td>
<td>190 ± 11</td>
<td>481 ± 25</td>
<td>8.1 ± 0.2</td>
<td>61.6 ± 6.6**</td>
<td>1.9 ± 0.2</td>
<td>0.83 ± 0.01**</td>
</tr>
</tbody>
</table>

BW, body weight; SBP, systolic blood pressure; HR, heart rate; KW, kidney weight; U-Alb, urinary albumin excretion; U-Na, urinary sodium excretion; Ccr, creatinine clearance. DL, non-nephrectomized rats (low salt); DH, non-nephrectomized rats (high salt); Nx-Sham, sham-operated nephrectomized rats (high salt); Nx-RDNx, renal denervated nephrectomized rats (high salt).

* \( P < 0.05 \) vs DH.

**P < 0.05** vs Nx-Sham.
In this *in vivo* study, we found that denervation resulted in local renal sympathoinhibition. Moreover, our data demonstrated that RDNx decreased urinary albumin excretion and reduced glomerular sclerosis and podocyte injury in Dahl salt-sensitive hypertensive rats. We also showed that the mechanism responsible for this improvement lay in the inhibition of intraglomerular ROS via NAD(P)H oxidase activation.

Inflammation and oxidative stress are risk factors for progressive renal dysfunction [25]. Veelken *et al.* suggested that renal denervation protects kidney function through an anti-inflammatory effect [26]. They reported that denervation significantly reduced albuminuria, mesangiolysis, formation of microaneurysms, deposition of glomerular collagen IV and expression of transforming growth factor-β in rat experimental Thy1.1 glomerulonephritis. We have previously reported that excessive oxidative stress exacerbates glomerular damage in an experimental rat diabetic model [22] and in a subtotal nephrectomized rat model [13]. These results suggest that ROS in glomeruli is a major cause of progressive glomerular injury, and ROS reduction would be very important for renal protection. From the present study, it appears that RDNx protects renal function and improves glomerular injury through reduced intraglomerular ROS. In the Dahl salt-sensitive hy-
pertensive rat, intrarenal oxidative stress is accelerated, thereby amplifying renal damage [27]. Excess glomerular ROS may induce endothelial dysfunction, which causes endothelial nitric oxide synthase uncoupling [22]. RDNx might improve the nitric oxide/ROS balance by improving glomerular endothelial function and thereby contribute to renal protection from hypertensive damage.

It has been reported that the sympathetic nervous system contributes to the level of renal NAD(P)H oxidase activity [28]. In sympathectomized spontaneously hypertensive rats, cortical and medullary NAD(P)H oxidase activities and renal vascular resistance were lower than in untreated spontaneously hypertensive rats. In the present study, we demonstrated that RDNx decreased glomerular NAD(P)H oxidase-derived ROS. There are several ways that RDNx may affect NAD(P)H oxidase activity. RDNx may reduce intraglomerular pressure, and, as a result, NAD(P)H oxidase activity may decline. Glomerular hypertension may increase the shear stress in glomerular endothelial cells. Rho-like small GTPase rac1, a key component of NAD(P)H oxidase activation, is activated by shear stress in endothelial cells [29]. Previous reports have demonstrated that increased renal SNA induced renin release, which increased intraglomerular pressure [30,31]. Therefore, RDNx would reduce intraglomerular pressure through local RAS suppression. Our data suggested that RDNx could not suppress the expression of AT1R and ACE mRNA in glomeruli, but plasma renin activity was suppressed in Nx-RDNx rats. Therefore, RDNx might reduce intraglomerular pressure through RAS suppression. Generally, plasma renin activity was suppressed by a high-salt diet in Dahl salt-sensitive rats. Thus, inhibition of RAS may not be the main mechanism of protection of glomerular function in this study.

In this study, we demonstrated that RDNx reduced ROS in glomeruli. This suggests that increased renal SNA may be associated with ROS production in glomeruli. Sympathetic nerves are involved in the regulation of contraction in glomerular afferent and efferent arteries, which determines intraglomerular pressure. Denton et al. investigated the effects of renal SNA on pre- and post-glomerular vascular resistance in rabbits [10]. They showed that the increase in renal SNA by moderate hypoxia induced contraction of the glomerular efferent artery and increased the glomerular filtration ratio. In the present study, RDNx attenuated intraglomerular pressure by blockade of renal SNA. However, RDNx did not reduce blood pressure in this model. Thus, RDNx may not be sufficient for long-term renoprotection because several fac-

Fig. 5. Glomerular expression of mRNA for the components of NAD(P)H oxidase. The mRNA levels of p47phox (A), p67phox (B), gp91phox (C) and p22phox (D) in rats that were not nephrectomized and underwent a sham operation (DH), that were nephrectomized (Nx-Sham) or that underwent renal denervation (Nx-RDNx) were measured by quantitative real-time PCR. Cytosolic components (p47phox and p67phox) were decreased in the Nx-RDNx group. n = 10 in each group. *P < 0.05 vs DH. †P < 0.05 vs Nx-Sham.

Fig. 6. Glomerular expression of the rac1 protein. Western blot shows that rac1 protein expression was lower in glomeruli from the renal denervation (Nx-RDNx) group than from the sham-operated group (Nx-Sham). n = 10 in each group. *P < 0.05 vs DH. †P < 0.05 vs Nx-Sham.
tors (such as hypertension and inflammation) affect progressive renal dysfunction.

Renal SNA increases the tone of renal blood vessels and stimulates renin release, thus attenuating renal tubular reabsorption [18,32]. In general, RDNx increases sodium excretion. It has been reported that RDNx prevented glomerular hyperfiltration by increased urinary sodium excretion in a model of diabetic nephropathy [33]. In the present study using Dahl salt-sensitive rats, sodium excretion was increased in the early phase of hypertension. However, in the late phase there was no difference in urinary sodium excretion between the sham-operated and RDNx groups. Blood pressure was not decreased by RDNx. Indeed, the salt-sensitive state was not affected by RDNx in this model. These results suggest that the increased sodium excretion is not associated with the reduction of intraglomerular pressure in this model, in contrast to the diabetic nephropathy model.

SNA directly induces ROS production. Several reports have shown that catecholamines increased directly NAD(P)H oxidase activity by stimulating α1- and β2-receptors [34,35]. Moreover, a β1-receptor antagonist improved endothelial function and reduced vascular oxidative stress by NAD(P)H oxidase [36]. In our ex vivo study using isolated glomeruli, only the β1-receptor antagonist stimulated NAD(P)H oxidase activity. These results suggest that there are more β1-receptors than α1- and β2-receptors in glomeruli [37]. Thus, RDNx may reduce glomerular ROS through inhibition of β1-receptor-mediated NAD(P)H oxidase activity.

**Fig. 7.** Renin–angiotensin system in glomeruli and systemic renin activity. (A) mRNA expression of angiotensin II type 1 receptor (AT1) detected by quantitative real-time PCR. AT1 mRNA expression in glomeruli was not significantly different in each group. (B) mRNA expression of ACE detected by quantitative real-time PCR. There was a significant increase in ACE mRNA expression between the DH and Nx-Sham or Nx-RDNx groups but no significant difference between the Nx-Sham and Nx-RDNx groups. *P < 0.05 vs DH. (C) Systemic renin activity. The renin activity was increased by nephrectomy. After RDNx, the activity was significantly decreased. *P < 0.05 vs DH.

**Fig. 8.** Ex vivo, catecholamine agonists stimulate NAD(P)H oxidase activity in glomeruli. (A) Glomerular NAD(P)H oxidase activity after stimulation of catecholamine agonists. The α1-receptor agonist phenylephrine (10−6 M) and the β2-receptor agonist salbutamol (10−6 M) did not stimulate NAD(P)H oxidase activity in isolated glomeruli, whereas the β1-receptor agonist dobutamine (10−7 M) stimulated NAD(P)H oxidase activity, relative to control (con), n = 6 in each group. *P < 0.05 vs con. (B) Glomerular NAD(P)H oxidase activity after stimulation of β1-receptor agonist. Stimulation of NAD(P)H oxidase by the β1-receptor agonist was dose dependent (10−7 to 10−5 M), and the β1-antagonist atenolol (10−7 M) suppressed NAD(P)H oxidase activity. n = 6 in each group. *P < 0.05 vs con. †P < 0.05 vs 10−5 M β1-receptor agonist.
Denervation reduces glomerular injury in Dahl rats

There is much clinical evidence to show that drugs that reduce SNA have a renoprotective effect. The results of the GEMINI study showed that carvedilol reduced albuminuria in patients with diabetes and hypertension [12]. Moxonidine, which decreases sympathetic nerve activity, was shown to have renoprotective effects in patients as well as in experimental rats with renal failure [38–40]. Moxonidine reduced microalbuminuria in patients with essential hypertension and diabetes [39]. Additionally, in a recent study, catheter-based renal sympathetic denervation was performed for resistant hypertension [41]. However, there have been few studies of the mechanisms involved in the renoprotective action of anti-SNA therapy. The results of the present study suggest one possible mechanism: the suppression of NAD(P)H oxidase activity in glomeruli through β1-receptor blockade. However, central SNA antagonists have several adverse effects, including depression and liver dysfunction, and therefore these drugs are not useful clinically. Dihydropyridine-type calcium channel blockers (CCBs) have been recommended as second-line antihypertensive therapy in CKD patients because of their potent antihypertensive effects. Several CCBs decrease heart rate by suppression of SNA. This suggests that selecting antihypertensive drugs with an effect on SNA is important for renal protection.

In conclusion, we have demonstrated that RDNx has a protective effect on kidney function in Dahl salt-sensitive rats, and this effect is associated with reduced oxidative stress and suppression of NAD(P)H oxidase activity. Thus, RDNx may exert its renoprotective effects by reducing SNA, thereby suppressing NAD(P)H oxidase activity.

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Conflict of interest statement. None declared.

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JNK signalling in human and experimental renal ischaemia/reperfusion injury

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Abstract

Background. Ischaemia/reperfusion (I/R) is an important factor in delayed graft function in renal transplantation and is a determinant of long-term graft outcome. This study examined the role of c-Jun N-terminal kinase (JNK) signalling in human and experimental renal I/R injury.

Methods. Biopsies obtained 15–20 min after reperfusion of human renal allografts were examined for JNK signalling by immunostaining for phospho-c-Jun. To examine the pathologic role of JNK signalling, a selective JNK inhibitor (CC-401) was administered to rats before or after the induction of a 30-min period of bilateral renal ischaemia followed by reperfusion. Renal function and tubular damage were analysed.

Results. Substantial JNK activation was evident in tubular epithelial cells in kidneys from deceased donors (n = 30) which was less prominent in kidneys from live donors (n = 7) (44.6 ± 24.8% vs 29.1 ± 20% p-c-Jun+, respectively; P < 0.05), whereas biopsies of thin basement membrane disease exhibited little, or no, p-c-Jun staining. The degree of p-c-Jun staining correlated with ischaemic time in deceased donor allografts, but not with graft function. Administration of CC-401 to rats prior to bilateral renal I/R prevented acute renal failure and largely prevented tubular damage, leucocyte infiltration and upregulation of pro-inflammatory molecules. However, delaying CC-401 treatment until 1 h after reperfusion (after the peak of JNK activation) had no protective effect.

Conclusions. We have identified acute activation of the JNK signalling pathway following I/R in human kidney allografts. Experimental studies indicate that blockade of JNK signalling, commenced prior to this activation, can prevent acute tubular necrosis and renal dysfunction secondary to I/R injury.

Keywords: apoptosis; c-Jun; ischaemia/reperfusion; kidney transplant; macrophage

Introduction

Ischaemia/reperfusion (I/R) injury is a significant clinical problem that occurs in a variety of diverse scenarios including kidney transplantation. It is an important cause...