Introduction

Ischemic acute kidney injury is often observed after transplantation of the kidney and also recognized as a major complication in cardiac and vascular surgery, septic and hypovolemic shock, and trauma (1). It is well known that renal ischemia/reperfusion associated with these clinical situations is responsible for ischemic acute kidney injury. Although many intracellular events have been shown to contribute to renal cell injury in ischemic acute kidney injury (2), the detailed mechanisms of the ischemia/reperfusion-induced ischemic acute kidney injury still remain unclear.

Patients with chronic kidney injury show high serum noradrenaline concentration that may result from an increase in sympathetic nerve activity (3). Animal experiments have shown that renal sympathetic nerve activity increased after renal ischemia, and inhibition of this nerve activation could prevent the development of ischemia/reperfusion-induced acute kidney injury (4, 5). It is important to note that renal application of noradrenaline produced ischemic acute kidney injury (6). These indications suggest exaggerated renal sympathoexcitation causes the renal dysfunctions. Recently, we reported that tyrosine hydroxylase expression in the renal tissue was increased in acute kidney injury, which was mediated by the central opioidergic system (7). Since blockade of central opioidergic systems improved the renal function in acute kidney injury model (7), it is possible that increased expression of tyrosine hydroxylase in the kidney plays a key role in the progression of ischemic kidney injury.

Although the renal sympathetic nerve supply and its distribution in normal conditions have been the subject of detailed morphological investigations (8, 9), systematic studies of nerve distribution in ischemic acute kidney injury kidney have not been performed. We reasoned that if renal ischemia/reperfusion-induced acute kidney injury

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is really due to renal sympathetic nerve activation, drastic neuronal changes should occur in ischemic acute kidney injury. To address these questions, we investigated that changes in renal sympathetic nerve supply using biochemical (western blotting) and morphological (immunohistochemistry) techniques. We further examined the influence of disturbing noradrenergic neurons by pharmacological tools on the physiological changes in ischemic acute kidney injury in mice.

Materials and Methods

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Kyushu University of Health and Welfare, as adopted by Committee on Animal Research of Kyushu University of Health and Welfare, which are accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Animals

Male ICR mice (SLC Japan, Inc., Shizuoka) weighing 20–30 g were used in this study. Animals were housed 5 per cage in a room maintained at 23°C ± 0.5°C with an alternating 12-h light-dark cycle. Food and water were available ad libitum. Animals were used only once in all experiments.

Acute kidney injury was induced by clamping both renal pedicles for 30 min with a sterile surgical procedure under pentobarbital anesthesia (60 mg/kg, i.p.). At the end of the ischemic period, the clamps were released for reperfusion. In sham-operated animals, both kidneys were treated identically without clamping renal pedicles. Twenty-four hours after the ischemia/reperfusion, blood samples were drawn from the abdominal aorta to evaluate renal function. The plasma samples were prepared by centrifugation. Blood urea nitrogen and plasma creatinine levels were measured using commercial assay kits, the BUN-test-Wako and Creatinine-test-Wako (Wako Pure Chemicals, Osaka), respectively.

Western blotting

The kidney was removed quickly after decapitation of mice and homogenized in ice-cold buffered sucrose solution containing 20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 0.5 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride plus 250 μg/ml leupeptin, 250 μg/ml apro tin, and 0.32 M sucrose. The homogenate was then centrifuged at 1,000 × g for 10 min at 4°C, and the resulting supernatant was used as protein samples for western blot analysis. The protein concentration was measured using a Bradford assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The same amount of protein samples was diluted with an equal volume of 2 × electrophoresis sample buffer containing 2% SDS and 10% glycerol with 0.2 M dithiothreitol. Proteins (25 μg) were separated by SDS-PAGE (12%) using buffer system of Laemmli (10). After electrophoresis, proteins were transferred to a nitrocellulose membrane (Bio-Rad Laboratories) in Tris/glycine buffer containing 25 mM Tris, 192 mM glycine, and 20% methanol. The membrane was soaked in a blocking buffer [3% bovine serum albumin (Sigma) in Tris-buffered saline (pH 7.6) containing 0.05% Tween-20 (TBS/T)] for 1 h at room temperature. The membrane was washed with TBS/T for three 10-min intervals. For detection of TH, the membrane was incubated with the mouse monoclonal anti TH (1:2000; Chemicon, Temecula, CA, USA) for overnight at 4°C with gentle agitation. The membrane was then washed in TBS/T, three 10-min intervals, before being incubated in horse-radish peroxidase–conjugated goat anti-rabbit IgG (1:20,000; Cell Signaling Technology Inc., Beverly, MA, USA) for 1.5 h at room temperature. After three 5-min washes in TBS/T and two 5-min washes in TBS, the antigen-antibody peroxidase complex was detected by enhanced chemiluminescence (Pierce) and visualized using VersaDoc 5000 (Bio-Rad Laboratories). The intensity of the band was analyzed and semiquantified by computer-assisted densitometry using Quality One software (Bio-Rad Laboratories).

Immunohistochemistry

Twenty-four hours after the renal ischemia/reperfusion, mice were anesthetized with pentobarbital (60 mg/kg, i.p.) and were intracardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS. Both side kidneys were removed, post-fixed in the same fixative for 2 h, and immersed in 30% sucrose/PBS solution overnight. Thick colateral sections of the middle of kidneys were initially dissected. Then, they were frozen in an embedding compound (Jung; Leica Microsystems, Heidenberg, Germany). Kidney sections of 8 μm (for single staining) or 25 μm (for double staining) were cut with the use of a cryostat (CM1900, Leica Microsystems) and thaw mounted on poly-L-lysine-coated glass slides.

In single staining procedures, kidney sections were blocked in 10% normal donkey serum in 0.1 M PBS for 2 h at room temperature. Each primary antibody was diluted in 0.1 M PBS containing 0.5% bovine serum albumin and 0.4% Triton-X 100 [1:500 goat anti tyrosine hydroxylase (TH; Santa Cruz biotechnology, Santa Cruz, CA, USA)] and was incubated for 2 days overnight at 4°C. The sections were then rinsed and incubated with the secondary antibody conjugated with Alexa 488 (Life Technologies Japan, Inc., Tokyo) for 2 h at room tem-
perature. The slides were then coverslipped with fluorescence mounting medium (Dako, Carpinteria, CA, USA). Fluorescence of immunolabeling was detected using a fluorescence microscope (AxioImager A1; Carl Zeiss, Jena, Germany).

The procedure for double-labeling tissue sections with tyrosine hydroxylase-antiserum and synapsin I-antiserum is as follows: Kidney sections were first blocked with 10% normal donkey serum (in 0.1 M PBS, 0.5% BSA, 0.4% Triton X-100) and then incubated in tyrosine hydroxylase-antiserum (1:500) for 2 days overnight at 4°C. The sections were then rinsed and incubated with the secondary antibody conjugated with Alexa-488 for 2 h at room temperature. After thorough rinsing with PBS for 1 h, tissues were blocked with normal goat serum, and incubated in rabbit polyclonal synapsin I-antiserum (1:250 dilution; Sigma-Aldrich, St. Louis, MO, USA) for 2 days overnight at 4°C. After washing with PBS for 30 min, tissues were incubated in Alexa-597–conjugated goat anti-rabbit IgG (1:400) for 2 h. Sections were washed for 30 min with PBS and then coverslipped with fluorescence mounting medium (Dako).

Sections were examined on a laser scanning confocal microscope (LMS 510 META, Carl Zeiss) with excitation/emission wavelengths 488/520 nm for Alexa-488 and 543/620 nm for Alexa-597.

**Lesions of noradrenaline neurons**

Lesions of noradrenaline neurons were performed by injection of the noradrenaline-selective neurotoxin DSP-4 (Sigma-Aldrich). According to the previous report for the complete denervation of noradrenaline neurons in the brain, a dose of 50 mg/kg of DSP-4 was injected subcutaneously and mice were received ischemia/reper-

fusion treatment 72 h later (11). This pretreatment time of DSP-4 has been shown 70% selective loss of noradrenaline contents in the cortex.

![Fig. 1. Expression of tyrosine hydroxylase immunoreactivity (TH-ir) in juxtaglomerular region of sham-operated (A) and renal ischemia-reperfusion (I/R, B) groups. Kidneys were removed at 24 h after the reperfusion. In the sham-operated group, TH-ir was found in only juxtaglomerular region. Arrows indicate the glomerulus. Renal I/R induced massive upregulation of TH-ir in the glomerulus in addition to juxtaglomerular region. Scale bar: 20 μm.](image1)

![Fig. 2. Confocal images of mouse renal glomerulus double-labeled with tyrosine hydroxylase- and synapsin I-antiserum. Sections double labeled with tyrosine hydroxylase (TH, A) and synapsin I (B) in renal tissue from 24 h after renal ischemia/reperfusion. In juxtaglomeruli and glomerulus, colocalization is shown by yellow dots in the merged images of TH/synapsin I (C). Scale bars (A, B, and C): 20 μm.](image2)
**Statistical analysis**

The data were expressed as the mean ± S.E.M. The statistical significance of differences between the groups was assessed with the Newman-Keuls multiple comparison test using GraphPad Prism version 3.0 software (GraphPad Software, San Diego, CA, USA). *P* < 0.05 was considered significant.

**Results**

**Immunohistochemical approach for the determination of tyrosine hydroxylase in the mouse kidney**

Figure 1 illustrates representative images of tyrosine hydroxylase immunoreactivity in the mouse kidney. The tyrosine hydroxylase immunoreactivity was observed around the renal tubule and juxtaglomerular region both in sham-operated and ischemic acute kidney injury mice (Fig. 1A). The tyrosine hydroxylase immunoreactivity was observed in the glomeruli of ischemic acute kidney injury mice (Fig. 1B), although tyrosine hydroxylase immunoreactivity could not be observed in the glomeruli of sham-operated mice.

To determine the cellular localization of tyrosine hydroxylase-immunoreactivity, kidney sections from 3 ischemic acute kidney injury mice were double-labeled with tyrosine hydroxylase and synapsin I antiserum. A synaptic terminal marker synapsin I-immunoreactivity in the kidney of ischemic acute kidney injury mice was observed in the juxtaglomerular region and glomeruli (Fig. 2A). Double-labeling experiments showed that tyrosine hydroxylase immunoreactivity was highly colocalized with synapsin I immunoreactivity in the juxtaglomerular region and glomeruli (Fig. 2C), suggesting new nerve terminals are formed in glomeruli after renal ischemia/reperfusion.

**Effects of DSP-4 on ischemia/reperfusion-induced increase of tyrosine hydroxylase expression in the mouse kidney**

As shown in Fig. 3A, renal tyrosine hydroxylase expression was remarkably increased after bilateral renal pedicle clamping for 30 min and 24 h of reperfusion compared with the sham-operated group, suggesting that progressive increase in renal noradrenaline synthesis takes place in ischemic acute kidney injury mice. Subcutaneous pretreatment with DSP-4 (50 mg/kg) 72 h before renal ischemia/reperfusion significantly inhibited ischemia/reperfusion-induced enhancement of renal tyrosine hydroxylase expression (Fig. 3). The basal expression of renal tyrosine hydroxylase was not affected by subcutaneous pretreatment with DSP-4 (Fig. 3).

**Effects of DSP-4 on renal function after ischemia/reperfusion**

Both blood urea nitrogen and plasma creatinine were remarkably elevated after bilateral renal pedicle clamping for 30 min and 24 h of reperfusion compared with the sham-operated group (Fig. 4). Subcutaneous (s.c.) pretreatment with DSP-4 at the dose of 50 mg/kg 72 h before renal ischemia/reperfusion significantly attenuated the renal ischemia/reperfusion-induced elevation of blood urea nitrogen and plasma creatinine (Fig. 4). Subcutaneous pretreatment with DSP-4 alone did not affect the blood urea nitrogen and plasma creatinine in the sham-operated group.
Acute Kidney Injury and Noradrenaline

Effects of DSP-4 on increased expression of glomerular tyrosine hydroxylase after ischemia/reperfusion

As shown in Fig. 5B, tyrosine hydroxylase immunoreactivity in the glomerular region was markedly intense after renal ischemia/reperfusion compared with the sham-operated group (Fig. 5A). Subcutaneous treatment with DSP-4 at the dose of 50 mg/kg 72 h before renal ischemia/reperfusion suppressed the change in the intense tyrosine hydroxylase immunoreactivity in the glomerular region (Fig. 5D). We could not detect obvious changes of tyrosine hydroxylase immunoreactivity after DSP-4 treatment in the sham-operated group (Fig. 5C).

Discussion

Activation of renal sympathetic nerve is thought to play an important role in the progression of ischemic kidney injury (3 – 5). The biochemical and anatomical changes of the sympathetic nervous system in ischemic acute kidney injury kidneys, however, are poorly understood. In the present study, we observed that tyrosine hydroxylase expression, which is related to the rate of noradrenaline synthesis and content in sympathetic nerves (12), prominently increased in the kidney of ischemia/reperfusion-induced ischemic acute kidney injury mice (Fig. 2). Moreover, localization of renal tyrosine hydroxylase-immunoreactivity in the ischemic acute kidney injury mice was different from that in the sham-operated mice: Tyrosine hydroxylase-immunoreactivity was expressed inside of the glomeruli only in the ischemic acute kidney injury (Fig. 1). These results further support the hypothesis that renal sympathetic nerve activity is increased in ischemic acute kidney injury. We also observed that pharmacological denervation of noradrenaline nerves by DSP-4 inhibited the ischemia/reperfusion-induced renal dysfunction evaluated by changes in blood urea nitrogen and serum creatinine (Fig. 4) and structural changes (Fig. 5) in ischemic acute kidney injury mice.

Renal ischemia is an important primary event leading to renal sympathetic nerve activation. Renal ischemia has been shown to activate the renal sympathetic nerves via stimulation of renal chemoreceptor (13). Renal ischemia causes an acute increase in sympathetic nerve activity, which is mediated through the increased afferent nerve activity from the kidney (14). In the present study, we observed that the expression of renal tyrosine hydroxylase was increased after ischemia/reperfusion (Fig. 3). These results lend further support to the hypothesis that the sympathetic nerve supply for the kidney increases after renal ischemia/reperfusion. The relationship between the progression of renal disease and the sympathetic nerve excitation has been well investigated (4, 15), and blockade of sympathoexcitation by moxonidine improved the pathophysiology of subtotally nephrectomized rats (15) and diabetic nephropathy patients (16). In ischemic acute kidney injury, pharmacological and surgical approaches for inhibiting renal sympathetic nerves prevented ischemia/reperfusion-induced renal dysfunction in rats (4). L-Carnosine prevented elevation of renal noradrenaline outflow and attenuated renal dysfunction induced by ischemia/reperfusion in rats (17). Our present observation indicates that denervation of noradrenaline nerves by DSP-4 attenuates ischemia/reperfusion-induced renal dysfunction (Fig. 4). These results provide the evidence that inhibition of renal sympathoexcitation
prevents the progression of ischemic acute kidney injury.

The distribution of tyrosine hydroxylase immunoreactivity in the glomeruli of ischemic acute kidney injury mice was unexpected. In the normal kidney, we could not detect any tyrosine hydroxylase immunoreactivity–positive cells in the glomeruli. No adrenergic fiber penetration into the glomeruli has been reported (18). Sympathetic nerves innervate the afferent and efferent juxtaglomerular arterioles and the interlobular arteries in the outer cortex of rabbit kidney and the afferent arterioles in the rat kidney (8). The tyrosine hydroxylase immunoreactivity is detected in the renal pelvic wall (19). In the present study, the glomerular tyrosine hydroxylase immunoreactivity was colocalized with synapsin I, a nerve terminal marker protein (Fig. 2). This colocalization of tyrosine hydroxylase with synapsin I implicates new synapse formation in the glomeruli after renal ischemia/reperfusion. It is therefore hypothesized that the synaptogenesis in the glomeruli is one of the reasons for the development of ischemic acute kidney injury. Alternatively, it is also possible that existing nerve terminals increases their expression of tyrosine hydroxylase. Although it is not clear whether synaptogenesis or increased expression in nerve terminals is involved in this increased tyrosine hydroxylase in glomeruli, in support of our present hypothesis, myocar-
dial ischemia/reperfusion is reported to induce cardiac sympathetic nerve sprouting that may contribute to the cardiac sudden death and arrhythmogenesis in myocardial infarction (20, 21).

The functional meaning of the sprouting adrenergic nerve into the glomeruli is not clear at this time. Podocytes, key cells in the genesis of glomerular injury (22), express α1- and β2-adrenoceptors (23). Activation of sympathetic nerves or application of noradrenaline decreases the glomerular diameter (24) and contracts podocyte foot process (25). Inhibition of the renal sympathetic nerve activity improves glomerulosclerosis in subtotaly nephrectomized rats (15) and microalbuminuria in type I diabetic mellitus patients (16). Chronic renal injury induced by ischemia/reperfusion has been shown to associate with the glomerulosclerosis (26). Thus, it is possible that the sprouting adrenergic nerve into the glomeruli after ischemia/reperfusion is involved in the development of chronic nephropathy such as diabetes and ischemic acute kidney injury. To clarify this hypothesis, further study is still required for greater certainly.

Our present results support the previous findings that the increased noradrenaline overflow is involved in renal ischemia/reperfusion-induced severe lesions in the kidney. Increased tyrosine hydroxylase activity in the glomerulus might be involved in the increased
noradrenaline concentration in renal venous plasma as reported previously (4). The increased adrenergic terminals in the glomerulus after renal ischemia/reperfusion could reduce glomerular functions and thereby affect the functional loss of tubules and vessels in the kidney. In the present study, treatment with DSP-4 attenuated the severity of renal dysfunction after renal ischemia/reperfusion (Fig. 4). We also indicated that DSP-4 treatment prevented the increase in expression of glomerular tyrosine hydroxylase immunoreactivity after renal ischemia/reperfusion (Fig. 5). Therefore, it is possible that increased adrenergic nerve terminals in the glomerulus indirectly obscures the tubular and vascular functions in acute kidney injury.

In conclusion, our present study suggests that renal ischemia/reperfusion produces the functional changes of renal noradrenergic systems, which play an important role in the development of ischemic acute kidney injury. Although additional investigations will need to uncover the precise pathways and intracellular events, the present results lead us to assume that functional changes of renal noradrenergic nerve terminal, especially in glomeruli, are involved in the several renal diseases including ischemic acute kidney injury.

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References