Tempol Protects against Ischemic Acute Renal Failure by Inhibiting Renal Noradrenaline Overflow and Endothelin-1 Overproduction

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Ischemic acute renal failure (ARF) is a frequent clinical syndrome with high morbidity and mortality.1) Reperfusion of previously ischemic renal tissue initiates a complex cellular events that results in injury and the eventual death of renal cells due to a combination of apoptosis and necrosis.2) The molecular mechanisms underlying the ischemia/reperfusion-induced renal injury are poorly understood, but it has been reported that several causal factors (ATP depletion, re-active oxygen species, phospholipase activation, neutrophil infiltration, vasoactive peptides etc.) are contributive to the pathogenesis of this renal damage.3) Enhancement of renal sympathetic nerve activity and its consequent effect on noradrenaline (NA) overflow from the nerve endings has also been considered as one of the factors which cause the ischemia/reperfusion-induced renal injury.4,5) In a recent study, we found that ischemia/reperfusion-induced ARF was attenuated by a surgical or pharmacological blockade of renal sympathetic nerve, followed by a suppression of elevated renal venous NA levels.6)

4-Hydroxy-2,2,6,6-tetramethyl piperidinoxyl (tempol) is a membrane-permeable and metal-independent superoxide dismutase mimetic that has been shown to be specific for superoxide anion (O$_{2}^{-}$).7,8) Several studies have demonstrated that tempol could reduce the renal dysfunction and injury caused by ischemia/reperfusion, mainly through its free radical scavenging activity.9,10) It is accumulating evidence that oxidative stress is one of causal factors in developing various cardiovascular diseases and that antioxidant agents and substances can attenuate cardiovascular diseases such as hyper-tension and post-ischaemic organ damage.11,12) In addition, recent studies have shown that tempol-induced reduction in O$_{2}^{-}$ production leads to decreased activity of renal sympathetic nerve, which may be contributive to the hypotensive action of tempol.13,14) These findings led us to evaluate the relationship between tempol-induced improvement on the post-ischemic ARF and renal sympathetic nervous system.

In the present study, we investigated the effect of tempol treatment on ischemia/reperfusion-induced renal dysfunction, tissue injury and NA levels in renal venous plasma, which are elevated in the post-ischemic kidney and involved in the post-ischaemic ARF.4—6) In addition, we examined the effect of tempol on renal endothelin-1 (ET-1) overproduction, which is a possible mediator of the pathogenesis of the post-ischaemic ARF.15—17)

MATERIALS AND METHODS

Animals and Experimental Design Male Sprague–Dawley rats (10 weeks of age, Japan SLC, Shizuoka, Japan) were used. Animals were housed in a light-controlled room with a 12 h light/dark cycle and were allowed ad libitum access to food and water. Experimental protocols and animal care methods in the experiments were approved by the Experimental Animal Committee at Osaka University of Pharmaceutical Sciences. Two weeks before the study (at 8 weeks of age), the right kidney was removed through a small flank incision under pentobarbital anesthesia (50 mg/kg, i.p.). After a 2-week recovery period, these rats were separated into five groups: (1) vehicle-treated sham-operated control (sham), (2) sham + tempol treatment (100 mg/kg, i.v.), (3) vehicle-treated ARF, (4) ARF + tempol treatment (10 mg/kg, i.v.), (5) ARF + tempol treatment (100 mg/kg, i.v.). To induce ischemic ARF, rats were anesthetized with pentobarbital (50 mg/kg, i.p.), and the left kidney was exposed through a small flank incision. The left renal artery and vein were occluded with a nontraumatic clamp for 45 min. At the end of the ischemic period, the clamp was released to allow reperfusion. Tempol or vehicle (0.9% saline) was injected 5 min before the start of ischemia, in a volume of 1 ml/kg into the external jugular vein. In sham-operated control rats, the kidney was treated identically, except for the clamping.

Animals exposed to 45-min ischemia were housed in...
metabolic cages at 24 h after reperfusion and 5-h urine samples were collected. Under pentobarbital anesthesia (50 mg/kg, i.p.), renal venous blood samples were obtained via a curved 26-gauge needle connected to a polyethylene catheter which was inserted into the left renal vein, and then arterial blood samples were drawn from the thoracic aorta. Finally, the left kidneys were excised. Each plasma was separated by centrifugation and used for measurements of NA concentrations and renal function parameters, respectively.

Analytical Procedures Blood urea nitrogen (BUN) and creatinine levels in plasma or urine were determined using commercial kits, the BUN-test-Wako and Creatinine-test-Wako (Wako Pure Chemical Industries, Osaka, Japan), respectively. Urinary osmolality was measured by freezing point depression (Fiske Associates, Norwood, MA). Urine and plasma sodium concentrations were determined using a flame photometer (Hitachi, 205D, Hitachinaka, Japan). Fractional excretion of sodium (FENa) was calculated from the following formula: \( \text{FENa} = \frac{\text{UNa} \times \text{Cr}}{\text{PNa} \times \text{Ccr}} \times 100 \), where \( \text{UNa} \) is urinary excretion of sodium, \( \text{PNa} \) is the plasma sodium concentration, and \( \text{Ccr} \) is creatinine clearance. Nor-epinephrine concentration in renal venous plasma was measured by high-performance liquid chromatography with an amperometric detector (EC-100, EICOM, Kyoto, Japan), as previously reported.18)

Histological Studies The excised kidneys were preserved in phosphate-buffered 10% formalin, embedded in paraffin wax, cut into thin sections (4 μm) according to conventional techniques. The sections were stained with hematoxylin and eosin. Histopathological changes were analyzed for tubular necrosis, proteinaceous casts, and medullary congestion, as suggested by Solez et al.19) Tubular necrosis and proteinaceous casts were graded as follows: no damage (0), mild (+1, unicellular, patchy isolated damage), moderate (+2, damage less than 25%), severe (+3, damage between 25 and 50%), and very severe (+4, more than 50% damage). The degree of medullary congestion was defined by: no congestion (0), mild (+1, vascular congestion with identification of erythrocytes by ×400 magnification), moderate (+2, vascular congestion with identification of erythrocytes by ×200 magnification), severe (+3, vascular congestion with identification of erythrocytes by ×100 magnification), and very severe (+4, vascular congestion with identification of erythrocytes by ×40 magnification). Evaluations were made in a blind manner.

Renal ET-1 Assay Endothelin-1 was extracted from the kidney, as described elsewhere.20) Briefly, kidneys were weighed and homogenized for 60 s in 8 volumes of ice-cold organic solution (chloroform/methanol, 2:1, including 1 mM \( N \)-ethylmaleimide). The homogenates were left overnight at 4°C, then 0.4 volumes of distilled water was added after which the homogenates were centrifuged at 1500 g for 30 min and the resultant was stored. Aliquots of the supernatant were diluted 1:10 with a 0.09% trifluoroacetic acid solution and applied to Sep-Pak C18 cartridges. The sample was eluted with 3 ml of 63.3% acetonitrile and 0.1% trifluoroacetic acid in water. Eluates were dried in a centrifugal concentrator, and the dried residue was reconstituted in assay buffer for radioimmunoassay. The clear solution was subjected to radioimmunoassay. The recovery of endothelin-1 was approximately 80%. Radioimmunoassay for tissue endothelin-1 was done, as described elsewhere,21) using endothelin-1 antiserum (a generous gift from Dr. Marvin R. Brown, Department of Medicine, University of California, San Diego, CA). This serum dose not cross-react with big endothelin-1.

Statistical Analysis Values were expressed as means ± S.E.M. Statistical analysis for renal functional studies was performed using one-way analysis of variance followed by a Dunnett-type multiple comparison test. Histological data were analyzed using Kruskal–Wallis nonparametric test combined with a Steel-type multiple comparison test. For all comparisons, differences were considered significant at \( p<0.05 \).

RESULTS

Renal Function after the Ischemia/Reperfusion and Effect of Tempol Treatment As shown in Table 1, renal function of rats subjected to 45-min ischemia showed a marked deterioration when measured 1 d after the reperfusion. As compared with sham-operated rats, vehicle-treated ARF rats showed marked increases in BUN, plasma creatinine concentration (Pcr) and FENa, and decreases in Ccr. Administration of tempol (10 or 100 mg/kg, i.v.) dose dependently attenuated the ischemia/reperfusion-induced renal dysfunction. Higher dose of tempol (100 mg/kg, i.v.) to sham-operated animals produced no effects in their renal functional parameters.

Histological Renal Damage after the Ischemia/Reperfusion and Effect of Tempol Treatment Histopathological examination revealed severe lesions in the kidney of vehicle-treated ARF rats (1 d after the ischemia/reperfusion). These changes were characterized by tubular necrosis in the outer zone outer stripe of medulla, medullary congestion and hemorrhage in the outer zone inner stripe of medulla and proteinaceous casts in tubuli in the inner zone of medulla. Administration of tempol (10 or 100 mg/kg, i.v.) attenuated the

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>BUN (mg/dl)</th>
<th>Pcr (mg/dl)</th>
<th>Ccr (ml/min/kg)</th>
<th>FENa (%)</th>
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<tbody>
<tr>
<td>Sham + vehicle (n=6)</td>
<td>23.4±1.3**</td>
<td>0.77±0.02**</td>
<td>3.54±0.45**</td>
<td>0.29±0.07**</td>
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<td>Sham + tempol, 100 mg/kg (n=6)</td>
<td>23.5±1.5**</td>
<td>0.71±0.04**</td>
<td>3.98±0.24**</td>
<td>0.44±0.09**</td>
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<tr>
<td>ARF + vehicle (n=6)</td>
<td>106.8±9.8</td>
<td>2.52±0.25</td>
<td>1.39±0.21</td>
<td>1.34±0.26</td>
</tr>
<tr>
<td>ARF + tempol, 10 mg/kg (n=6)</td>
<td>74.4±7.1*</td>
<td>1.84±0.19*</td>
<td>2.09±0.30</td>
<td>0.95±0.18</td>
</tr>
<tr>
<td>ARF + tempol, 100 mg/kg (n=6)</td>
<td>66.9±8.2**</td>
<td>1.52±0.16**</td>
<td>2.33±0.21*</td>
<td>0.61±0.14**</td>
</tr>
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</table>

Data are the mean±S.E.M. ∗p<0.05, **p<0.01, compared with ARF+Vehicle. BUN, blood urea nitrogen; Pcr, plasma creatinine; Ccr, creatinine clearance; FENa, fractional excretion of sodium; ARF, acute renal failure.
development of all these lesions in a dose-dependent manner (Fig. 1). Sham-operated control animals showed no lesions (data not shown).

Renal Venous Plasma NA Concentration after the Ischemia/Reperfusion and Effect of Tempol Treatment

It has been considered that NA overflow into the renal vein is useful to assess the activity of renal sympathetic nervous system. Since the ischemia/reperfusion-induced renal injury was attenuated by a surgical or pharmacological blockade of renal sympathetic nerve, followed by a suppression of elevated renal venous NA levels, it is likely that the enhancement of renal sympathetic nerve activity is closely related to the development of the post-ischemic ARF. Therefore, we measured renal venous plasma NA concentrations after the ischemia/reperfusion, with or without tempol treatment. As shown in Fig. 2, NA levels in vehicle-treated ARF rats were remarkably elevated, compared with those in sham-operated control animals. Administration of tempol (10 or 100 mg/kg, i.v.) dose-dependently suppressed the ischemia/reperfusion-induced increments of renal ET-1 content.

DISCUSSION

It is well acknowledged that, in ischaemia/reperfusion-induced renal injury, reperfusion is essential for the survival of ischaemic cells, but reperfusion itself causes additional tissue injury. This reperfusion injury appears to be attributed to the generation of reactive oxygen species (ROS) such as O$_2^·$ and hydroxyl radical, and hydrogen peroxide.23,24 This view is supported by findings that anti-oxidative enzymes and ROS scavengers can improve the ischemia/reperfusion-induced ARF.

Tempol is a stable, membrane-permeable, metal-independent superoxide dismutase mimetic that has been shown to be specific for superoxide anion (O$_2^·$), and reduces superoxide-related injury in post-ischaemic cardiac damage, hypertension, inflammation, and radiation. In the ischaemia/reperfusion-induced acute ARF, tempol is reported to attenuate renal dysfunction by protecting tubular (mainly proximal tubular cells) cells from oxidative stress. We also found that renal dysfunction in ARF rats exposed to 45-min ischaemia and reperfusion was markedly improved by the treatment with tempol in a dose-dependent manner. Histological damage such as tubular necrosis, proteinaceous casts in tubuli and congestion/hemorrhage, in medullary region, was also significantly suppressed by the tempol treatment.

In the present study, we asked whether the renal sympathetic nerve activity is related to the pathogenesis of ischaemia/reperfusion-induced renal injury and its improvement by tempol. To attain this, we determined NA levels in renal venous plasma. Results clearly indicated that the NA levels were elevated after the ischemia/reperfusion, suggesting an enhancement of NA overflow from the kidney exposed to the ischaemia/reperfusion. Tempol could markedly suppress the elevation of NA levels. We have recently demonstrated
that the elevation of renal venous NA levels after the ischemia/reperfusion was suppressed by renal denervation or ganglion blocker, both of which ameliorated renal dysfunction and tissue injury of the kidney exposed to ischemia/reperfusion. It has been reported that acute administration of tempol (30—50 mg/kg, i.v.) to normotensive or hypertensive rats causes a decrease in renal sympathetic nervous system activity, accompanied by reductions of blood pressure and heart rate. In addition, since an inhibitor of superoxide dismutase (diethyldithio-carbamic acid) significantly increased blood pressure, heart rate and neural activity of renal sympathetic nerve, Shokoji et al. suggested that the tempol-induced hypotensive effect are due to a reduction of renal sympathetic nerve activity and that augmented O$_2^-$ production contributes to the development of hypertension through activation of the sympathetic nervous system. Taken together with the view that NA overflow into the renal vein is useful to assess the activity of renal sympathetic nervous system, it is reasonable to consider that the sympatheoinhibitory effect of tempol is at least partly responsible for its protective effect on the ischaemia/reperfusion-induced renal injury.

Recently, Xu et al. demonstrated that tempol-induced inhibition of sympathetic nerve activity and depressor action were not accompanied by superoxide dismutase mimetic action in vascular tissues, suggesting a direct sympathoinhibitory effect of tempol. We observed that O$_2^-$ production was markedly increased in the kidney 1 d after 45-min ischemia followed by the reperfusion, and tempol administration revealed only slight suppressive action on the above increment (Fujii et al., unpublished observations). In order to clarify whether tempol-induced improvement on the post-ischemic renal injury and NA overflow is directly linked to its superoxide dismutase mimetic action, further studies at histochemical level are needed.

It is acknowledged that ET-1 is closely related to the development of the ischemia/reperfusion-induced ARF. Renal ET-1 mRNA expression and tissue content of ET-1 peptide are elevated in the postischemic kidney. Both selective endothelin ETA and nonselective endothelin ET$_A$/ET$_B$ receptor antagonists have been indicated to improve the ischemia/reperfusion-induced renal dysfunction. Thus, the up-regulation of renal ET-1 production and its ETA receptor-mediated action are likely to contribute to the pathogenesis of ischemic ARF. In the present study, we investigated the effect of tempol on ET-1 overproduction in the kidney subjected to ischemia/reperfusion. Although the effects of the lower dose (10 mg/kg) were not statistically significant, the higher dose (100 mg/kg) reduced the increased ET-1 content in postischaemic kidneys to levels seen in sham-operated control rats. Thus, the beneficial effects of the higher dose of tempol on renal dysfunction and tissue injury in ischemic ARF are associated with suppression of ET-1 overproduction induced by the ischemia/reperfusion. Although the mechanisms by which ET-1 production is enhanced in the kidney of ischemic ARF are obscure, there are several reports indicating that the ET-1 gene expression and the peptide production are up-regulated under the hypoxic condition, both in tubular cells and endothelial cells. Oxidative stress is known to stimulate renal ET-1 production at a stage of its gene expression. Moreover, some antioxidative agents could suppress the ET-1 production in renal and vascular endothelial cells. Taken together, it seems likely that tempol can suppress the ischemia/reperfusion-induced renal ET-1 overproduction through its antioxidative activity. However, it remains to be determined where ET-1 overproduction following the ischemia/reperfusion occurs (in vascular endothelium, tubular cells or possibly in both). One available evidence is that ET-1 is first expressed in increased quantities in the peritubular capillary network shortly after the onset of renal ischemia and then transported across the basement membrane of the adjacent tubular epithelial cell, which are then sloughed off during the development of acute tubular necrosis.

Since changes in renal ET-1 contents were parallel to those in renal venous NA levels, one might speculate that ET-1 overproduction in the post-ischemic kidney resulted from the enhanced NA release from renal sympathetic nerves. In a recent study, we found that ischemia/reperfusion-induced ARF was attenuated by a surgical or pharmacological blockade of renal sympathetic nerve, followed by a suppression of elevated renal venous NA levels. However, the increment of ET-1 content was not suppressed by the same blockade (unpublished observations). Thus, it seems likely that the renal ET-1 overproduction does not depend on the enhanced renal sympathetic nervous activity.

In conclusion, preischemic treatment with tempol overcame the ischemia/reperfusion-induced renal injury. Inhibitory effects on the renal sympathetic nerve activity and ET-1 overproduction seems to be involved in the beneficial actions of tempol.

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REFERENCES


