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Effects of ulinastatin (urinary trypsin inhibitor) on ATP, intracellular pH, and intracellular sodium transients during ischemia and reperfusion in the rat kidney in vivo

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Abstract

Purpose. To investigate the effects of ulinastatin on renal ischemia–reperfusion injury, we monitored the dynamic changes in ATP, intracellular pH (pHi), and intracellular sodium (Nai) in rats in vivo.

Methods. Renal ischemia was induced by clamping the abdominal aorta for 30 min followed by reperfusion for 60 min. Ulinastatin, 50 000 U·kg^{-1} (UTI group), or normal saline (NS group) was infused for 30 min before ischemia. ^{31}P- and double quantum ^{23}Na-NMR were used to monitor ATP, pHi, and Nai.

Results. During ischemia, ATP was rapidly depleted and Nai increased to the same extent in both groups. After 60 min reperfusion, Nai in the NS group was almost restored to the preischemic baseline level (117.2 ± 7.4% of the baseline value), but the recovery of ATP was incomplete (60.9 ± 7.7%). The recovery of Nai in the UTI group began earlier than in the NS group with better recovery of ATP. The pHi values showed severe acidosis in the NS group compared with the UTI group during ischemia and reperfusion. As for ultrastructural findings, after 60 min reperfusion, the mitochondria were less swollen and less disorganized with respect to the membrane and the cristae in the UTI group.

Conclusion. The transcellular sodium gradient is restored before the ATP level is normalized during postischemic reperfusion. Ulinastatin might protect mitochondrial conformation during ischemia, and facilitate functional recovery of the ionic pump after reperfusion.

Key words Renal ischemia · Ulinastatin · Intracellular sodium · Intracellular pH · ATP

Introduction

Ischemic acute renal failure is one of the most common complications from aortic surgery. The incidence of postoperative renal failure requiring dialysis has been reported to be more than 10% of patients with aorta cross-clamping [1]. Insufficient blood flow causes anaerobic metabolism and depression of various ionic pumps in the membranes. After blood flow is reestablished, cellular dysfunction mediated via biochemical effectors and oxidative processes follows. Prior to the activation of such mediators, adenosine triphosphate (ATP), high-energy compounds, is consumed and depleted at the start of ischemia. It has previously been shown that ATP is rapidly depleted during ischemia, and that the percentage recovery of ATP varies with the duration of the ischemia [2,3]. ^{23}Na-NMR studies have shown that intracellular Na^+ rises to approach that in the external medium after prolonged ischemia in the perfused kidney [4]. Although these early alterations of such bioenergetic and ionic circumstances are thought to initiate ischemic cellular injury, intracellular Na^+ transients during ischemia and reperfusion have not been examined in the kidney in vivo.

Appropriate renal protection could prevent a prerenal syndrome developing into acute tubular necrosis. Ulinastatin (UTI; urinary trypsin inhibitor) is a Kunitz-type protease inhibitor that inhibits the activity or release of lysosomal enzymes such as elastase and catepsin G [5,6]. It has been reported to help protect against shock and operative stress [7–10] and suppress the deterioration of renal function associated with surgical procedures, chemotherapy, and various types of shock [11–13]. However, the effects of UTI on energy metabolism and intracellular Na^+ levels have not been fully investigated, and its mechanisms for protecting against renal ischemia have not yet been clarified.

In order to investigate the pathophysiology of ischemic shock at the cellular level of the kidney, we monitored the dynamic changes in ATP, intracellular pH, and intracellular Na^+ during ischemia and reperfusion in the rat in vivo using ^{31}P- and double quantum filtered (DQ) ^{23}Na-NMR, and also investigated the effects of UTI on the ischemic kidney. As the

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^{23}Na DQ technique utilizes only differences in the transverse relaxation of intra- and extracellular quadrupolar ions, the concentration of intracellular Na^+ could be measured without chemical shift agents [14,15]. We also examined alterations in the mitochondrial microstructure in tubular epithelial cells under an electron microscope.

Materials and methods

The study was approved by Kagawa Medical University animal investigation committee. Twenty-eight male Wistar rats (JCL–Wistar, Japan Clare, Hamamatsu, Japan; body weight 350–450 g) were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg·kg^{-1}). After tracheotomy, the lungs were ventilated with a ventilator for small animals (EVM-50A, Aika Medical, Tokyo, Japan). Anesthesia was maintained with isoflurane (0.7%). Polyethylene catheters (PE-50s, Clay-Adams, NJ, USA) were inserted into the right femoral artery for blood pressure monitoring and the right femoral vein for infusion. The left kidney was exposed through a dorsal incision. A vascular occluder (OC4A, In Vivo Metric Systems, Healdsburg, CA, USA) was placed around the abdominal aorta just above the bifurcation of the renal artery. Renal ischemia was achieved by inflating the balloon of the vascular occluder, and was confirmed by 0 mmHg of femoral arterial blood pressure. The kidney was placed on a five-turn surface coil (10 mm diameter) in the NMR probe. To minimize the NMR signal from surrounding tissues, a thin copper sheet was placed on tissues around the kidney. The rat was placed in the bore of the magnet in the vertical, head-up position. During the experimental period, the body temperature was maintained at 37.0 ± 0.5°C using a home-built temperature controller. Thirty minutes prior to ischemia, normal saline, 1 ml (NS group, $n = 14$), or ulinastatin, 50 000 U·kg^{-1} dissolved in 1 ml normal saline (UTI group, $n = 14$), was injected intravenously. UTI was donated by the Mochida Pharmaceutical Company (Tokyo, Japan). Both groups received an infusion of normal saline at 2 ml·h^{-1} during ischemia (30 min) and reperfusion (60 min) in both groups. Each group was divided into subgroups for ^{31}P-NMR measurements ($n = 7$) and ^{23}Na-NMR measurements ($n = 7$).

^{31}P-NMR measurements. ^{31}P-NMR spectra were obtained on a GSX 270 WB spectrometer (6.3T, JEOL, Tokyo, Japan) operating at 109.23 Mhz. The pulse width was 10 μs and 400 scans were accumulated at 1.0-s intervals. Since the β-peak of adenocine triphosphate (β-ATP) is the only peak that was unique to ATP on the spectrum, tissue ATP levels were assessed by comparing changes in the area of β-ATP. Intracellular pH (pHi) was calculated from the chemical shift difference (δ) between the pH-independent resonance of the α-ATP and the pH-dependent resonance of the inorganic phosphate (Pi) [16].

$$pHi = 6.75 + \log (\delta - 11.44)/(13.37 - \delta)$$

To ensure the stability of the experimental conditions, triplicate ^{31}P-NMR spectra of the kidneys were recorded to establish a baseline prior to initiating experimental procedures. The aorta was then clamped. ^{31}P-NMR spectra were measured three times (10-min intervals) during ischemia and four times (15-min intervals) during reperfusion.

^{23}Na-NMR measurements. ^{23}Na-NMR was conducted at 71.32 Mhz. ^{23}Na DQ NMR spectra were collected using the spin-echo DQ filter method described by Seo et al. [15]. The following pulse sequence was used: $90° - \tau/2 - 180° - \tau/2 - \Delta$ – acquisition. A DQ evolution time (Δ) of 0.03 ms was used. A creation time (τ) of 2 ms was used since this value had been shown to give almost maximum DQ signal intensity. Triplicate ^{23}Na DQ spectra were recorded to establish a baseline. ^{23}Na DQ spectra were then measured every 10 min during ischemia and reperfusion. Intracellular Na^+ (Nai) was assessed by comparing percentage changes from the mean control peaks in the signal heights of ^{23}Na DQ spectra.

At the end of the experiment, slices of renal tissue were obtained as quickly as possible, diced, and fixed for 1 h in osmium tetroxide. After fixation, tissues were dehydrated in ascending concentrations of ethanol, embedded in epon resin, and sectioned ultrathinly with a Solval ultramicrotome. They were then stained with urinyl acetate and lead citrate and examined under an electron microscope (1200EX, JEOL, Tokyo, Japan) by a pathologist with no knowledge of the experimental group to which the rat belonged.

All values are expressed as mean ± SE. Mean values for each group were compared by one-way analysis of variance (ANOVA). A P value less than 0.05 was considered to be statistically significant.

Results

Figures 1 and 2 show representative ^{31}P and ^{23}Na DQ NMR spectra, respectively, recorded prior to ischemia, after 30 min of ischemia, and after 60 min of reperfusion in the NS group. At the end of the ischemic period, the β-ATP signal disappeared and the Pi signal increased and shifted to the right. The recovery of the β-ATP signal and the Pi chemical shift after 60 min of reperfusion were incomplete. The ^{23}Na DQ signal intensity increased with a 30-min ischemic insult, and had

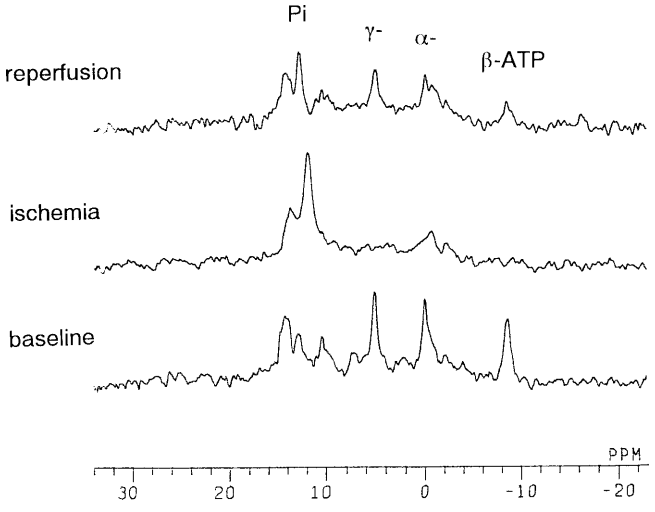

Fig. 1. Representative ^{31}P-NMR spectra of a rat kidney in the saline group after: baseline establishment; 30 min ischemia; 60 min reperfusion. *Pi*, inorganic phosphate; *ATP*, adenocine triphosphate

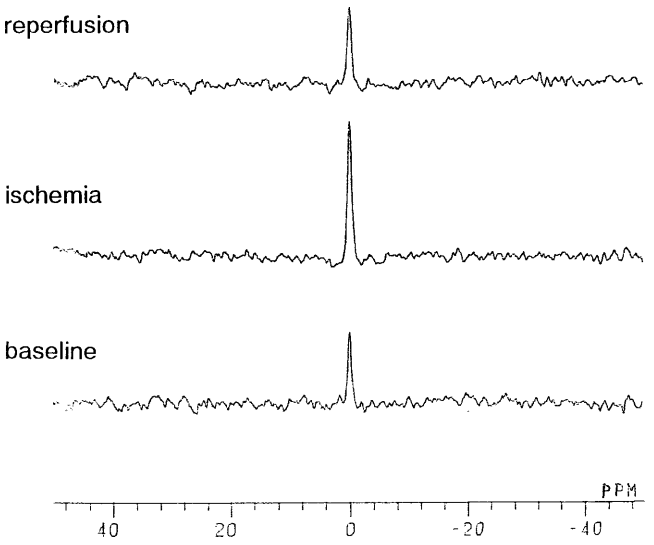

Fig. 2. Representative ^{23}Na double quantum filtered NMR spectra of a rat kidney in the saline group after: baseline establishment; 30 min ischemia; 60 min reperfusion

almost returned to baseline level at the end of the reperfusion period.

Figure 3a illustrates the time course changes in mean arterial pressure (MAP) in both groups. No significant difference was found between them throughout the course of the experiment.

ATP and pHi changes are illustrated in Fig. 3b and c, respectively. ATP was rapidly depleted during ischemia in both groups. However, during the reperfusion period, the recovery of ATP in the UTI group was faster and more complete than in the NS group. There was a significant difference after the 15 min reperfusion between the NS group (36.8 ± 5.4) and the UTI group (61.0 ± 6.0). Even after 60 min reperfusion, the recovery of ATP in each group was incomplete (NS, 60.9 ± 7.7; UTI, 73.0 ± 2.6). There was no significant difference in the basal pHi between the NS group (7.25 ± 0.03) and the UTI group (7.25 ± 0.02). In the NS group, pHi showed severe acidosis (6.35 ± 0.06) after 30 min ischemia, and also (7.07 ± 0.09) after 60 min reperfusion. In the UTI group, pHi was 6.53 ± 0.06 after 30 min ischemia, which was not as acidic as that in the NS group, and it finally returned to 7.23 ± 0.04 after 60 min reperfusion. The UTI group exhibited significantly higher pH values than the NS group from 10 min after the onset of ischemia to 30 min after initiation of reperfusion.

Nai changes are shown in Fig. 3d. Nai increased to 217.7 ± 24.6% of baseline level in the NS group and 232.7 ± 14.5% in the UTI group by the end of the ischemic period, with no significant difference between the groups. However, during the reperfusion period, Nai in the UTI group was restored faster than that in the NS group. There were significant differences between the groups from 30 min after initiation of the reperfusion period to the end of the experiments.

In an electron micrographic analysis (Fig. 4), the mitochondria in the NS group was swollen and disorganized with respect to the membrane and the cristae after 60 min reperfusion. In contrast, the morphological changes in mitochondria in the UTI group improved in comparison to those in the NS group.

Discussion

Our results indicated that Nai levels in both groups increased to more than 200% of baseline values during ischemia with the depletion of ATP. After reperfusion, the Nai level in the UTI group was restored faster than in the NS group. The proximal tubule and the medullary thick ascending loop of Henle contain Na^+-K^+ ATPase (Na^+ pump), are rich in mitochondria, and are extremely active metabolically. They are susceptible to ischemia because they consume the major fraction of the ATP in the kidney. Since the Na^+ pump on the cellular membrane is largely responsible for maintaining this ionic gradient, which regulates the reabsorption of various electrolytes and substances, the changes in intracellular Na^+ concentration might indicate dysfunction of the cellular membrane and of the renal tubular function itself. In the NS group, the depleted ATP was increased to 36.8% and 60.9% of the baseline value at 15 and 60 min, respectively, after initiation of

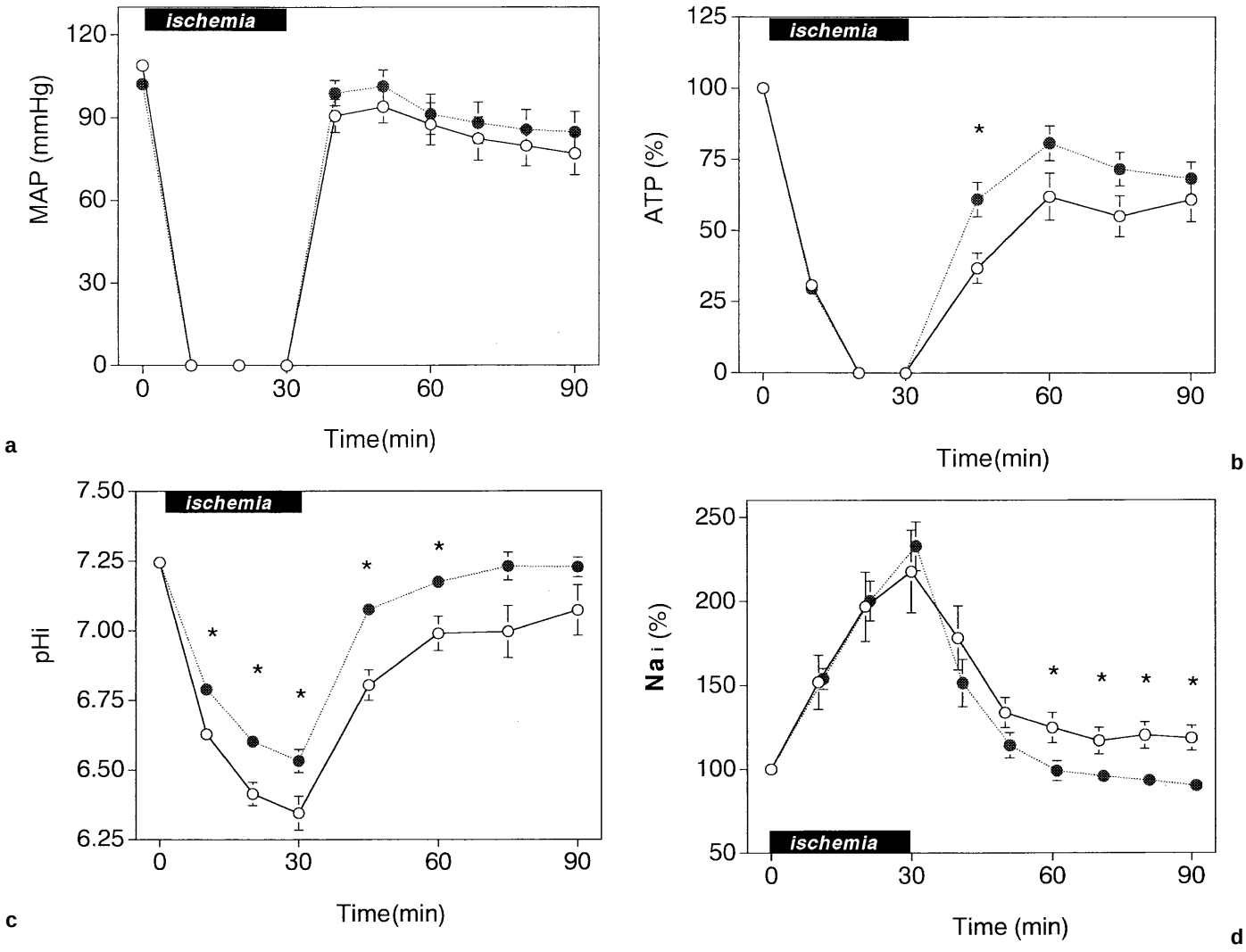

Fig. 3. Time course changes in **a** mean arterial pressure (MAP), **b** adenocine triphosphate (ATP), **c** intracellular pH (pHi), and **d** intracellular sodium (Nai) during the ischemia and reperfusion periods. Saline (*open circles*) or ulinastatin 50000 U·kg^{-1} (*closed circles*) was injected 30 min before onset of ischemia. ATP and Nai levels were assessed by comparing percentage changes from preischemic baseline values. All values are expressed as mean ± SE. $n = 7$ for each group. *Significant difference between the groups ($P < 0.05$)

reperfusion, whereas the augmented Nai declined to 133.8% and 117.2% of the baseline value after 20 and 60 min reperfusion, respectively. In the UTI group, ATP was 61.0% and 73.0% at 15 and 60 min of reperfusion, respectively, while Nai had almost recovered after 20 min reperfusion. Since the increased Nai almost returned to the baseline values within 90 min after ischemia in both groups, although only two-thirds of ATP had been restored, it is supposed that the ATP requirement to pump out the accumulated intracellular Na^+ is two-thirds of the normal ATP level in renal tubular cells. Eleff et al. [17] reported that intracellular Na^+ was fully recovered with 50% restoration of ATP in dog cerebral ischemia. The full recovery of ATP may be delayed due to the utilization of ATP for the restoration of the transcellular sodium gradient.

Intracellular acidosis due to the accumulation of lactate following anaerobic metabolism weakens the lysosomal membrane and activates various proteases in the lysosome. Moreover, oxygen free radicals produced during reperfusion after ischemia may contribute to the destruction of cellular membranes. UTI has been reported to have a protective effect against renal ischemia by the stabilization of the lysosomal membrane, the inhibition of lysosomal enzymes such as elastase and catepsin G [5,6], and its action as a free radical scavenger [6,18], but there have been no reports from the in vivo metabolic point of view. In this study, pHi, ATP,

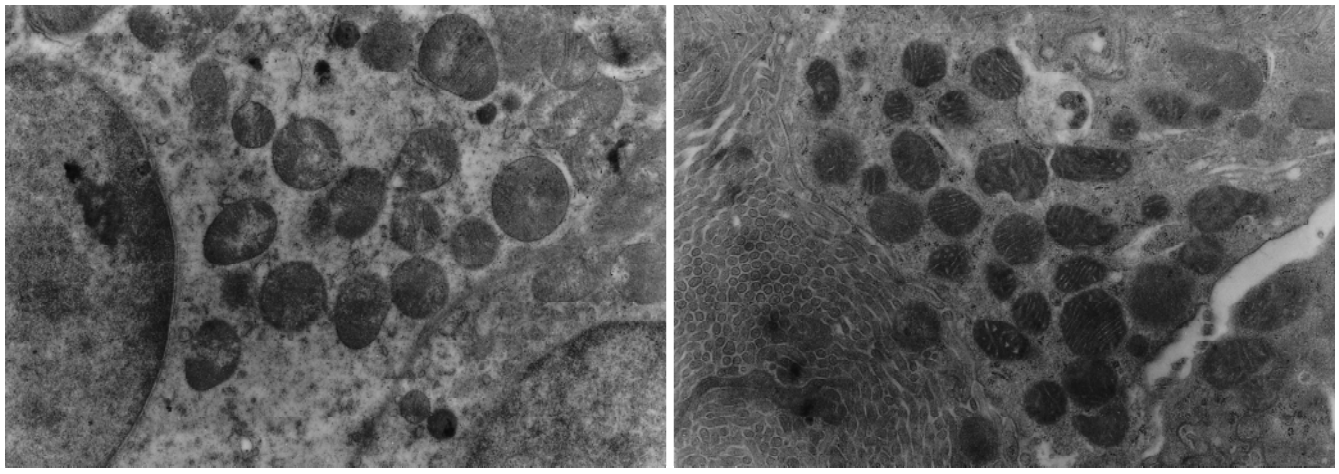

Fig. 4. Electron micrograph of renal tubular epithelial cells after a postischemic 60-min reperfusion period. (×10000) **a** Saline or **b** ulinastatin 50000 U·kg^{-1} was injected 30 min before ischemia

and Nai levels were restored faster in the UTI group than in the NS group after reperfusion under the same MAP. Because of the faster restoration of ATP in the UTI group, increased Nai levels due to the deterioration of ATP-dependent Na^+ pumps after early depletion of ATP were restored near to the baseline level, enabling the UTI group to recover faster than the NS group. However, it is not known whether the faster restoration of decreased pHi in the UTI group was due to the early restarting of the ionic pump function or to the reduced production of anaerobic metabolites during ischemia.

The disproportion between intra- and extracellular ions caused by dysfunction of ATP-dependent ionic pumps during ischemia is associated with cellular damage. An increase in intracellular Na^+ concentration induces morphological changes such as cellular swelling. Although Ishigami et al. [12] reported that the extent of tubular epithelial cell damage, as observed under light microscopy in cases of gentamicin- and $HgCl_2$-induced renal failure, was less in animals administered UTI, there have been no electron microscopic studies on the effect of UTI on renal ischemia. We observed conformational changes of mitochondria in relation to energy metabolism. After 60 min reperfusion, the mitochondria in the UTI group were less swollen and less disordered with respect to the membrane and internal structure than those in the NS group. Thus, the less damaged mitochondria in the UTI group also contributed to the restoration of depleted ATP faster than the much damaged ones in the NS group.

Mela et al. [19] suggested that released lysosomal enzymes in ischemic tissues might directly cause the mitochondrial impairment. It is also reported that oxygen free radicals produced during the reperfusion period injure mitochondria both directly and indirectly [20]. The protective effects of UTI on mitochondrial damage may be due to the inhibition of lysosomal enzyme release or activity and of free radical production. Recent studies suggested that UTI inhibits the production and release of cytokines such as tumor necrosis factor-α, and interleukin-6 and -8 [6,21]. Since UTI improved pHi and ATP during the ischemia and the early reperfusion periods in the present study, the protective effects of UTI on mitochondria and the Na^+ pump may not be directly concerned with the alterations in such cytokines. Further studies will be necessary to elucidate this mechanism.

In conclusion, the transcellular sodium gradient is restored before the ATP level is normalized during postischemic reperfusion in rat kidney. UTI protects the renal mitochondria from ischemia both functionally and morphologically, as well as restoring the transmembrane sodium gradient after reperfusion. UTI could prove useful for the protection of renal function from ischemic damage.

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