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Assessment of high-energy phosphorus compounds in the rat kidney by in situ ^{31}P nuclear magnetic resonance spectroscopy: effect of ischemia and furosemide

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Summary. ^{31}P nuclear magnetic resonance (NMR) spectroscopy of the in situ rat kidney was performed by a surface coil method, and the effects of ischemia and furosemide infusion were assessed. ^{31}P NMR spectra of the kidney subjected to 30 min of ischemia returned completely to the pre-ischemic level after 60 min of reperfusion. But the ^{31}P NMR spectra after 60 min of ischemia did not recover, even after 120 min of reperfusion. Levels of β-ATP and inorganic phosphate (Pi) decreased and the chemical shift of Pi increased after intravenous infusion of furosemide. This increase in chemical shift might signal an alkalotic change in intracellular pH. Furosemide infusion prior to ischemia is thought to protect the kidney from injury induced by 60 min of warm ischemia. The chemical shift of Pi returned to the pre-ischemic level earlier than β-ATP and Pi. In conclusion, according to the findings of ^{31}P NMR spectroscopy, furosemide infusion prior to ischemia may be effective in protecting the kidney against ischemic injury. But the change in Pi peak and the causes of the dissociation of Pi and β-ATP should be examined further.

Key words: Furosemide – Ischemia – Kidney – ^{31}P nuclear magnetic resonance spectroscopy – Surface coil

^{31}P nuclear magnetic resonance (NMR) spectroscopy is the only method currently available for measuring the metabolism of high-energy phosphorus compounds non-invasively and repeatedly [6]. This method has been utilized for several organs including the kidney in animal experiments [1, 3, 14]. Although localized ^{31}P NMR spectroscopy of brain, liver and muscle is feasible [11–13], that of the kidney is still almost impossible because of the motion artifact caused by respiration.

The viability of organs is a major problem in organ transplantation. The limit of warm ischemic time in the case of renal transplantation is said to be 30 min [5], and pre-ischemic infusion of furosemide or mannitol thought to be useful in protecting the kidney from ischemic injury [7]. However, little information is available about the effect of furosemide on high-energy phosphorus compounds in the ischemic kidney. Thus, in the present study we used in situ ^{31}P NMR spectroscopy under a high magnetic field to examine the change in high-energy phosphorus compounds occurring during ischemia and reperfusion, during furosemide administration, and during ischemia and reperfusion after the pre-ischemic infusion of furosemide.

Material and methods

Sixteen male Wistar rats (5 weeks old, 250–300 g body weight) were anesthetized with 50 mg/kg pentobarbiturate injected intraperitoneally, and the left kidney exposed by a dorsal incision. As it took approximately 30–60 min to prepare for ^{31}P NMR spectroscopy of the rat kidney (dissection of kidney, shimming, etc.), blood pressure remained stable and no side effect of the pentobarbiturate was present during the ^{31}P NMR spectroscopy.

The kidney was carefully dissected from the surrounding tissue, and the wound sutured so as not to occlude the renal vessels. A rubber pad was attached around the surface coil to avoid injury to the kidney and the rat was fixed to the surface coil, keeping the kidney in close contact with the surface coil through a thin polytetrafluoroethylene (PTFE) sheet. A 100-cm loop 5-0 catgut was made around the renal vessels, so that both ends of the catgut hung outside the spectrometer. A weight of 20–30 g was attached to the ends of the catgut to induce ischemia and was removed to permit reperfusion (Figs. 1–3). Control of renal blood flow by this method was confirmed by a hydrogen clearance tissue blood flow meter (MT Giken, Tokyo, Japan). The 16 rats were divided into the following four groups of 4 animals each:

Group 1: Kidneys were subjected to 30 min of warm ischemia followed by reperfusion.

Group 2: Kidneys were subjected to 60 min of warm ischemia followed by reperfusion.

Group 3: 5.0 mg/kg furosemide was injected intravenously into the rats without induction of ischemia.

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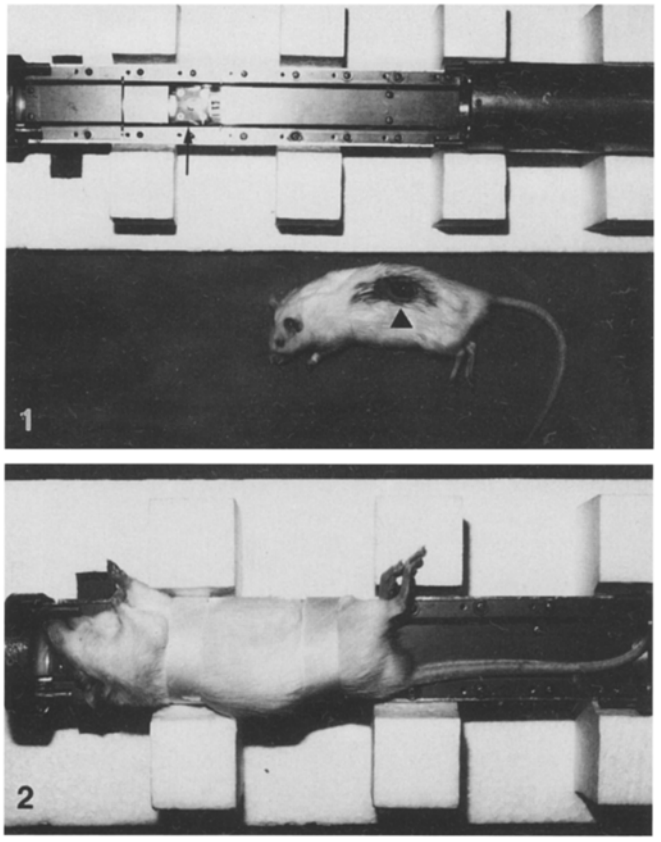

Fig. 1. Surface coil probe and rat. The surface coil was beneath the PTFE sheet (*arrow*). The left kidney of the rat was exposed through a dorsal incision (*arrowhead*)

Fig. 2. The rat was fixed to the surface coil, keeping the kidney in close contact with the surface coil through the PTFE sheet

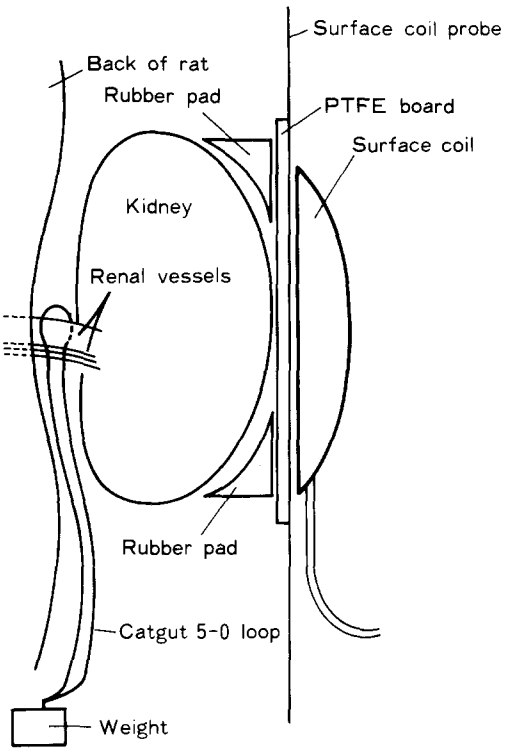

Fig. 3. Diagram of the kidney and surface coil

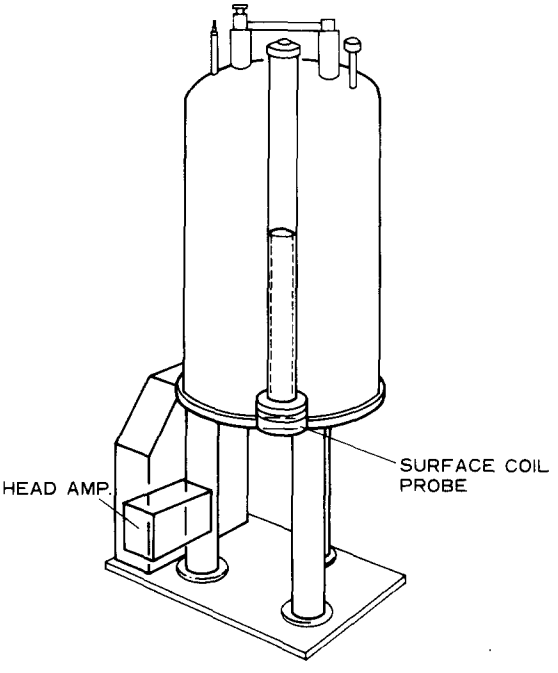

Fig. 4. Diagram of the JNM GX-270 spectrometer. The vertical bore is 5.4 cm in diameter

Group 4: Kidneys were subjected to 60 min of warm ischemia preceded by intravenous injection of 5.0 mg/kg furosemide and followed by reperfusion.

In groups 1, 2, and 4, ^{31}P NMR spectroscopy was performed for 2 h after the beginning of reperfusion.

^{31}P NMR spectroscopy of in situ rat kidney

Intracellular phosphorus metabolites were directly monitored by ^{31}P NMR spectroscopy in whole rat kidney with the surface coil. Spectra were obtained at 109.25 MHz on a JEOL spectrometer using a 6.34 T, 5.4-cm diameter vertical bore, superconducting magnet with a DEC mini-computer system (JNM GX-270, Nippon Densi, Tokyo, Japan). Magnetic field homogeneity was confirmed by 50 Hz of water half-line width. The water signal was used to shim the magnet by double-tuned circuit before acquiring the ^{31}P spectra. A surface coil (10 mm in diameter, made of copper wire) was utilized for spectroscopy, and a sealed capillary tube containing hexamethylephosphoroamide (HMPA) was placed on the other side of the surface coil as an external reference standard (Fig. 4).

Conditions for measurement

For the pulse sequence, pulsed NMR using the Fourier transformation technique was used. The pulse width was 15.0 μs, acquisition time 0.102 s, pulse delay time 1.900 s, frequency 10000 Hz, number of data points 2048, and scan time 200 times. These conditions of measurement, gave the best detection of signals from regions about 3 mm from the surface coil (namely the full thickness of the cortex and outer layer of the medulla of the kidney). Single spectroscopy took about 6 min. The spectra were obtained from the Fourier transformation of an acquired free induction decay (FID) pulse. Chemical shift was referenced to the HMPA peak, and the peak assignment described by Radda et al. [10] was used. Chemical shift

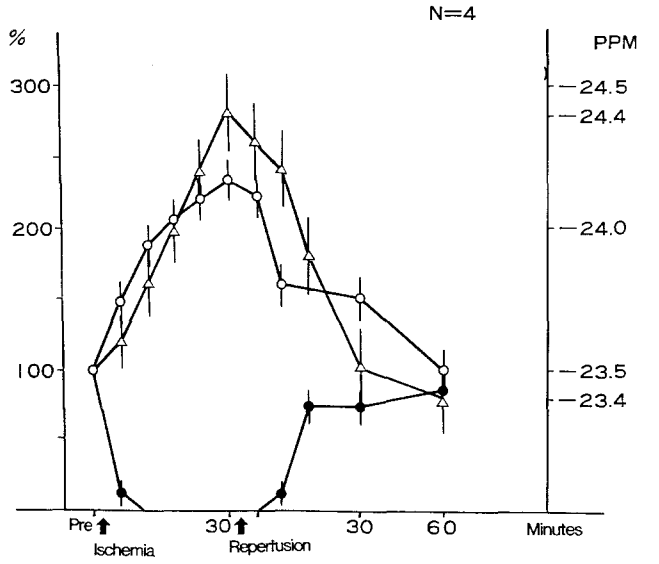

Fig. 5. Changes in ^{31}P spectra after 30 min of warm ischemia followed by reperfusion. ○, Pi (QPi); △, chemical shift of Pi (CHPi); ●, β-ATP (Qβ-ATP)

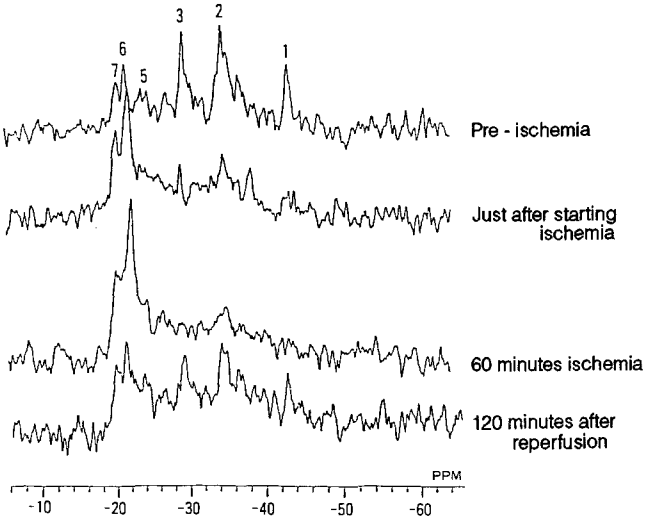

Fig. 6. Changes in ^{31}P spectra after ischemia and reperfusion. Assignment of peaks *1–7* is as follows: *1*, β-ATP; *2*, α-ATP + ADP; *3*, γ-ATP + ADP; *4*, creatinine phosphate, not seen; *5*, phosphodiester; *6*, Pi (inorganic phosphate); *7*, sugar phosphate (2,3-DPG, etc)

Table 1. Changes in Qβ-ATP

	Level of Qβ-ATP				
	Pre-ischemia %	60 min after reperfusion %		120 min after reperfusion %	
Group 1	100	91.5 ± 5.1	**		
Group 2	100	21.6 ± 4.3	*	44.2 ± 3.8	n.s.
Group 4	100	40.0 ± 5.8		9.3 ± 6.4	

* $P < 0.05$; ** $P < 0.01$
n.s., Not significant

Table 2. Changes in QPi

	Level of QPi				
	Before ischemia %	60 min after reperfusion %		120 min after reperfusion %	
Group 1	100	101.3 ± 10.5	**		
Group 2	100	171.1 ± 12.5	*	155.3 ± 10.9	*
Group 4	100	115.8 ± 11.4		107.9 ± 10.5	

* $P < 0.05$; ** $P < 0.01$

was calculated by the equation:

$$r = \frac{m_2 - m_1}{m_1} \times 10^6 \text{ ppm}$$

where r is chemical shift, and m_2 the frequency of pulse acquired from observed substance m_1 the frequency of pulse acquired from the reference substance.

Quantitative analysis of each peak (Q) was calculated from the ratio of the peak height of the observed peak (O) to the reference HMPA peak (R), that is Q = O/R. ^{31}P spectra of thigh muscle, brain and liver were also measured by the same system and the same pulse sequence, but the surface coil was directly attached to covering skin for comparison of the pattern of ^{31}P NMR spectra. Statistical analysis was performed using Student's *t*-test. All experiments were performed at room temperature. Urine flow of the kidney being examined could not be measured because urine was not produced for several hours or days after 30–60 min of warm ischemia. Measurement of precise intracellular pH could not be performed because the calibration curve of the change of Pi differ as a result of the changes in extracellular ions.

All animals were alive during these experiments and the data from every animal could be analyzed.

Results

Changes in ^{31}P spectra after warm ischemia followed by reperfusion

On *group 1* the ratio of peak height of Pi to HMPA (QPi) increased rapidly, chemical shift of Pi (CHPi) decreased rapidly and the ratio of peak height of β-ATP to HMPA (Q-β-ATP) decreased rapidly after ischemia. The peak of β-ATP could not be found after 12 min of warm ischemia. At the end of 30 min of warm ischemia, QPi was 2.8 times than the pre-ischemia value, and CHPi had decreased by 0.9 ppm. After reperfusion, QPi decreased and CHPi increased, reaching their pre-ischemic level after 60 min of reperfusion. In contrast, Qβ-ATP did not return to the pre-ischemic level (Fig. 5; Tables 1, 2).

On *group 2* the changes in spectra after ischemia and before reperfusion paralleled those in group 1. At the end

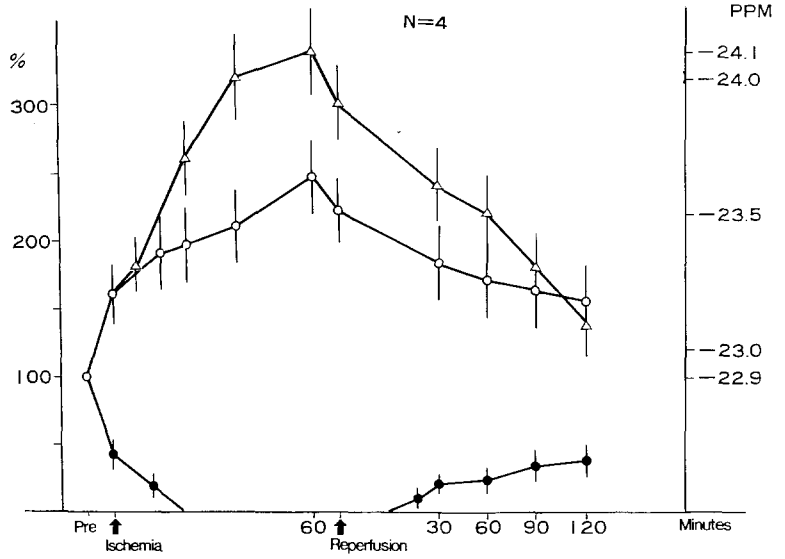

Fig. 7. Changes in ^{31}P spectra after 60 min of warm ischemia and reperfusion. ○, Pi (QPi); △, chemical shift of Pi (CHPi); ●, β-ATP (Qβ-ATP)

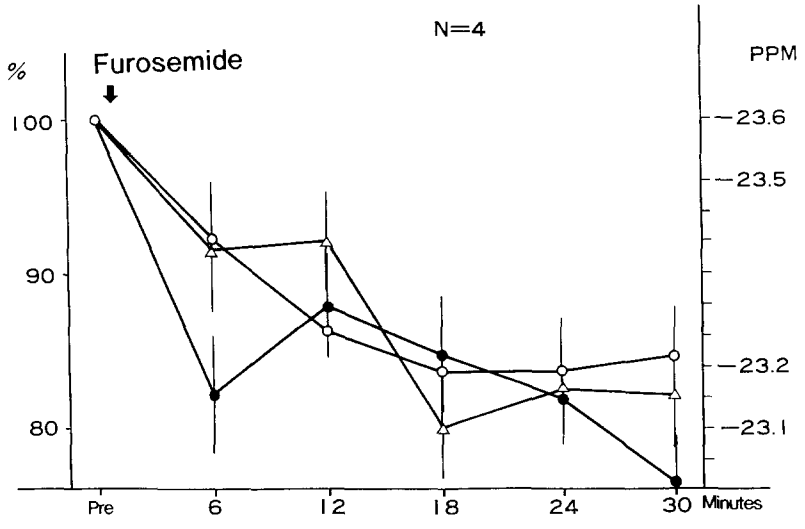

Fig. 8. Changes in ^{31}P spectra: effect of furosemide. ○, Pi (QPi); △, chemical shift of Pi (CHPi); ●, β-ATP (Qβ-ATP)

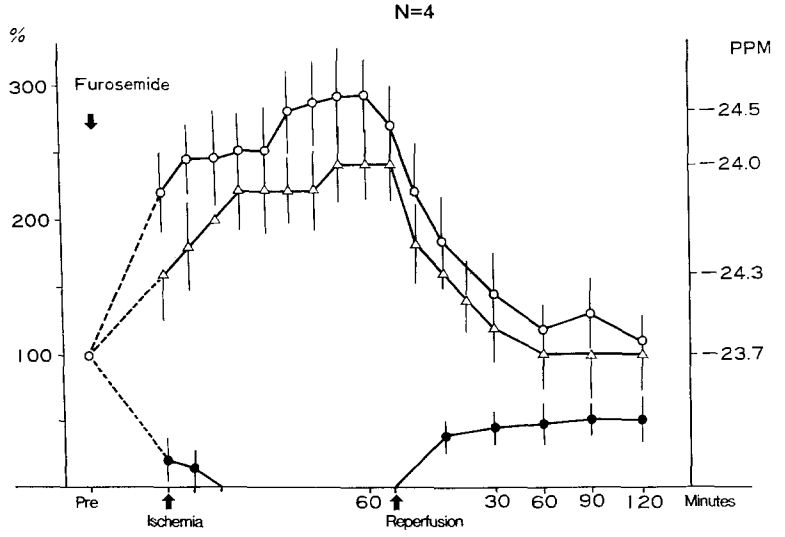

Fig. 9. Effect of furosemide on the changes in ^{31}P spectra: 60 min of warm ischemia followed by reperfusion. ○, Pi (QPi); △, chemical shift of Pi (CHPi); ●, β-ATP (Qβ-ATP)

of 60 min of ischemia QPi was 3.4 times greater than the pre-ischemic level and CHPi decreased by 1.2 ppm. After reperfusion, QPi decreased more slowly than in group 1 and did not return to the pre-ischemic level even after 120 min of reperfusion (Figs. 6, 7; Tables 1, 2).

On *group 3* changes in ^{31}P spectra were observed for about 30 min after intravenous infusion of 5.0 mg/kg furosemide. QPi and Qβ-ATP gradually decreased and CHPi increased, that is, cellular pH moved in an alkalotic direction (Fig. 8).

On *group 4* the changes in CHPi differed from those in group 2 (without furosemide). At the end of 60 min of warm ischemia, CHPi decreased by only 0.3 ppm and returned to the pre-ischemic level rapidly after reperfusion. QPi and Qβ-ATP also recovered more rapidly than in group 2 (Fig. 9; Tables 1, 2).

Discussion

There were three important findings in the present study. First, metabolism of high-energy phosphorus compounds by the in situ rat kidney recovered rapidly after reperfusion following 30 min of warm ischemia, but not following 60 min of warm ischemia. This reflects the common clinical experience that most kidneys transplanted after a warm ischemic time of 30 min or less function soon after transplantation.

Second, the level of β-ATP decreased and chemical shift of Pi increased after furosemide infusion for at least 30 min. This findings is consistent with the results of a high-performance liquid chromatography study in which the level of ATP in the rat kidney decreased after furosemide infusion [8]. It is thought that furosemide may reduce mitochondrial activity of renal cells and activate other metabolic pathways. Although many reports have been published on the change in intracellular pH measurement by ^{31}P NMR spectroscopy, we were not able to measure the intracellular pH. The reason for this is that the calibration curve of the Pi peak changed due to the change in extracellular ionic conditions. Comparing our results with the findings from other organs [11, 12], the intracellular pH of the kidney might become more alkaline after administration of furosemide.

Third, pre-ischemic infusion of furosemide resulted in an earlier recovery of the Pi level after reperfusion following 60 min of ischemia than when furosemide was not administered. The mechanisms involved in the protective effect of furosemide are unknown, but we suppose that furosemide reduces mitochondrial activity and renal cellular damage due to warm ischemia. Thus, it would be interesting to know what mitochondrial enzymes are blocked by furosemide.

Our results show one problem which originated from the experimental technique. At the end of the reperfusion experiment after 60 min of ischemia, β-ATP and Pi dissociated; this finding is incomprehensible. The cause of this dissociation may be the difficulty in confirming the Pi peak when the organ is in a well-oxygenated condition, and the fact that the Pi peak often overlaps the peaks of other phosphorus compounds (2,3-DPG and urinary Pi) [3].

With our method we were able to examine non-invasively the levels of and changes in high-energy phosphorus compounds in the in situ kidney. However, our method is limited by the fact that measurement of a very small area is impossible, and that injury to the kidney may not be avoidable despite the use of a rubber pad to cushion it.

Recently, the preliminary success of image-guided ^{31}P magnetic resonance spectroscopy of normal and transplanted human kidneys using ISIS (Image-Selected In vivo Spectroscopy) technique has been reported [2]. Although only invasive localized spectroscopy of animals is currently available, non-invasive volume-selected NMR spectroscopy of the renal cortex or medulla will find widespread application in the human kidney in the near future.

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