^{31}P Magnetic Resonance Spectroscopy of Perfused Rat Skeletal Muscle

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Abstract : Phosphocreatine (PCr), ATP, inorganic phosphate (P_i) and intracellular pH (pH_i) of biceps femoris of adult male rats were measured with phosphorus magnetic resonance spectroscopy (^{31}P-MRS). Isolated muscle was arterially perfused in an NMR tube with oxygenated Krebs-Henseleit bicarbonate solution at 37℃. PCr and pH_i were decreased during no-flow ischemia and P_i was increased, while ATP remained almost unchanged throughout the experiment. PCr and P_i were returned to the respective control levels by reperfusion. The pH_i decrease during ischemia was slower than the PCr decrease. When PCr was reduced to about a half of the control level, pH_i began to fall. Since the creatine-kinase catalyzed reaction (Lohmann's reaction) produces ATP with consuming H^+ but the glycolysis cannot dispose H^+, threshold of the pH_i fall may correspond to a turning point where the main source of the ATP production is switched from Lohmann's reaction to the glycolysis. These results suggest that PCr has a role of pH_i buffering during the early stage of ischemia in a typical fast-twitch muscle such as rat biceps femoris.

Key words : isolated skeletal muscle, arterial perfusion, ^{31}P-MRS, phosphocreatine, intracellular pH

INTRODUCTION

There are several clinical situations in which skeletal muscle is exposed to sustained ischemia followed by reperfusion such as during orthopedic trauma, sports injury, vascular and plastic surgery. Experience from clinical practice has shown that skeletal muscle can recover after several hours of ischemia without persistent damage (Heppenstall *et al.*, 1986). This could be partly explained by a low-energy turnover in resting muscle, but the tolerance to ischemia of skeletal muscle is probably different from that of other organs.

Phosphorus magnetic resonance spectroscopy (^{31}P-MRS) has been used to study metabolism of intact functioning skeletal muscle by measuring phosphocreatine (PCr), ATP and inorganic phosphate (P_i) concentrations and intracellular pH (pH_i) (Ingwall, 1982 ; Meyer *et al.*, 1982 ; Van den Thillart and Van Waarde, 1996 ; Roussel *et al.*, 2000). Dynamic aspects of these metabolites and pH can be defined simultaneously and nondestructively by using ^{31}P-MRS. *In vivo* ^{31}P-MRS using a surface coil was applied to the analysis of energy metabolism of skeletal muscle (Meyer and Adams, 1990 ; Foley *et al.*, 1991 ; Roman *et al.*, 1996 ; Harkema *et al.*, 1997). *In vivo* MRS studies with naturally or artificially ventilated animals take advantage to the evaluation of muscle metabolism under intrinsic neural and

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hormonal controls. However, surface coil is unavoidable for signal sampling from mixed muscles. Mammalian skeletal muscles are composed of three types of the fiber : fast glycolytic (type IIB), fast oxidative glycolytic (type IIA) and slow oxidative (type I) (Close, 1972 ; Ariano *et al*., 1973). These muscle fibers differ in their mechanical and metabolic characteristics, which reflect the differences in the contents of PCr, mitochondria, isoforms of enzymes and contractile proteins. We measured PCr, ATP, P_i and pH_i changes of rat biceps femoris with *in vivo* ^{31}P-MRS during ischemia by ligation of the muscle, and during hypoxia by ventilating with 10% O_2 gas (Uchida *et al*., 1996). pH_i did not fall until PCr was reduced to about 40% of the control value even in ischemia or hypoxia. However, in our *in vivo* ^{31}P-MRS experiments, we could not deny a risk to observe NMR signals from mixed muscles under a surface coil. Furthermore, it was difficult to analyze the time course of changes in the metabolites and pH_i during ischemia.

In contrast to *in vivo* ^{31}P-MRS, we can identify the measuring muscle with *in vitro* experiment using isolated muscle. Oxygenation of the isolated skeletal muscle is one of the most important points for *in vitro* ^{31}P-MRS experiments (Ruderman *et al*., 1971 ; Meyer and Adams, 1990 ; Kushmerick *et al*., 1992 ; Harkema *et al*., 1997). A superfusion method was used for *in vitro* ^{31}P-MRS of skeletal muscle at low temperatures (Dawson *et al*., 1977 ; Phillips *et al*., 1993 ; Zeleznikar *et al*., 1995). Superfusion is not sufficient for constant and prolonged oxygenation at physiological body temperatures of mammals. On the other hand, arterial perfusion is an ideal preparation for the *in vitro* MRS study. Adams *et al*. (1991), Kushmerick *et al*. (1983) and Meyer *et al*. (1985) developed a perfusion technique to ^{31}P-MRS and analyzed energy metabolism of cat skeletal muscles. Gutierrez *et al*. (1988) also reported perfusion ^{31}P-MRS for rabbit skeletal muscle. Besides of the advantage of the oxygenation and muscle identification, perfusion MRS is useful to simulate ischemia by stopping a perfusion pump.

Thus we designed a ^{31}P-MRS study of arterially perfused rat biceps femoris at the body temperature to investigate a possible role of PCr to buffer the pH_i decrease during ischemia. Rat biceps femoris is a typical fast-twitch muscle (Ariano *et al*., 1973) and rich in PCr. Perfusion ^{31}P-MRS of skeletal muscle has not been reported for small mammals such as a rat.

MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats (350－400g) were used. Animals were fed with standard laboratory chow and tap water *ad libitum*. The experiment was approved by the Animal Research Laboratory of Yamagata University School of Medicine.

Muscle preparation

The rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (6mg / 100g body wt). Skin incisions were made from thigh to ankle of either limb (usually left limb). The biceps femoris was easily identified because of its superficial location. The skin of the medial side was also removed because the femoral artery was situated in this side of the thigh. All superficial and loose areolar tissues were removed to cannulate the femoral artery. Branches of the femoral artery except for the biceps were ligated to perfuse only the biceps. The biceps femoris was carefully removed without damage to the muscle fibers. After the dissection, the veins draining the muscle were cut. Then, the femoral artery was cannulated with a cannula of 24 gauge. The muscle preparation is schematically shown in Fig.1. About 0.2ml of a heparin sodium solution (1,000IU / ml) was injected via the cannula to prevent intravascular

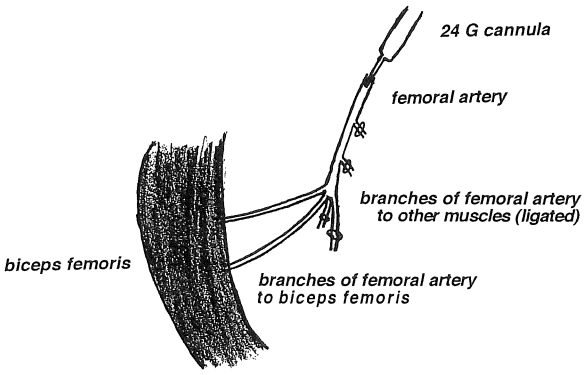

Fig. 1　Muscle preparation for the arterial perfusion

coagulation. The entire operative procedure required about 2 hours. During the operation the rat was artificially ventilated on a warmed plate, and the muscle and surrounding tissues were moistened with warm saline to prevent surface desiccation.

Perfusion method

The isolated biceps femoris was perfused in an NMR tube with a Krebs-Henseleit bicarbonate solution containing phenylphosphonic acid (PPA) (Fisher and Dillon, 1987). PPA was used as an external standard to evaluate the PCr, ATP and P_i concentrations. PPA was also used to estimate extracellular pH (pH_e). The perfusate consisted of 115.0 NaCl, 24.0 $NaHCO_3$, 3.8 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 10.0 glucose, 10.0 Hepes and 2.0 PPA in mM. Schematic drawing of apparatus used for the perfusion MRS is shown in Fig. 2. The NMR tube with a diameter of 25mm was specially designed and suitable to hold the isolated rat biceps femoris. The perfusion solution was pumped with a peristaltic pump from a reservoir warmed in a water bath. The temperature of the muscle was kept at 37℃ with a warmed perfusion solution and a temperature-controlling apparatus of the NMR system. The solution was passed through a silicone tubing in contact with a 95% O_2 / 5% CO_2 mixed gas. The Po_2 of the solution was confirmed to be nearly 600 Torr using a blood gas analyzer (Radiometer, ABL-50). The muscle was perfused from the femoral artery at a constant flow rate (0.12L / hr). No-flow ischemia was attained by switching off the perfusion pump.

NMR spectroscopy

^{31}P-MRS of the perfused rat biceps femoris was measured with a vertical type spectrometer (JEOL, Lambda-300) at a frequency of 121.55 MHz. The homogeneity of the magnetic field was initially optimized with superconductive shim coils. The pulse width was 40 μs. NMR spectra were obtained from free induction decays (FIDs) accumulated for 329 times with a repetition time of 0.91 s. This repetition time was sufficient for relaxation of the magnetic spins with a flip angle of 30 degree. The accumulated FIDs were multiplied by an exponential function corresponding to 10 Hz line broadening before the Fourier transformation. The NMR spectra were collected during control, no-flow ischemia and reperfusion periods. Relative intensity of PCr, β-ATP, P_i and PPA were measured as the respective peak area with a computer. Absolute concentrations of the metabolites were evaluated from the respective relative intensity as compared with that of PPA. The concentration of ATP was calculated from the peak area of β-ATP, which is free from overlapping with peaks of ADP or NADH.

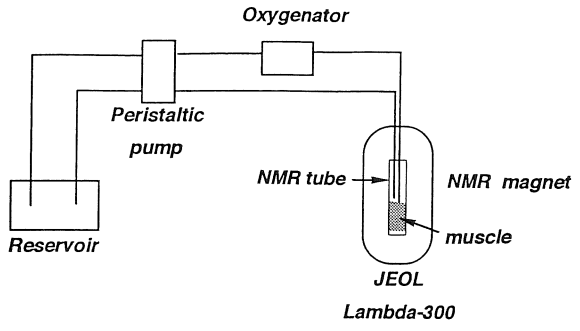

Fig. 2 Schematic drawing of apparatus used for the perfusion MRS experiments.

Intra- and extracellular pH

pH_i and pH_e were estimated as follows according to Meyer *et al.* (1985) and Fisher and Dillon (1987) :

$$pH_i = 6.77 + \log [(\delta_1 - 3.41) / (5.71 - \delta_1)], \quad (1)$$

where δ_1 is the chemical shift difference between P_i and PCr and

$$pH_e = 7.09 + \log [(17.39 - \delta_2) / (\delta_2 - 13.64)] \quad (2)$$

where δ_2 is the chemical shift difference between PPA and PCr.

Statistical analysis

Statistical evaluation between the control, no-flow ischemia and reperfusion groups was performed using one-way analysis of variance (ANOVA). All values are expressed as means ± SD, and the statistical significance was set at $P < 0.05$.

RESULTS

NMR spectra of the perfused muscle

Typical ^{31}P-MRS spectra of arterially perfused rat biceps femoris are shown in Fig. 3. Large PCr, weak P_i and moderate ATP peaks were observed in the control spectrum (Fig. 3A). PPA peak was well separated from these peaks and no significant change

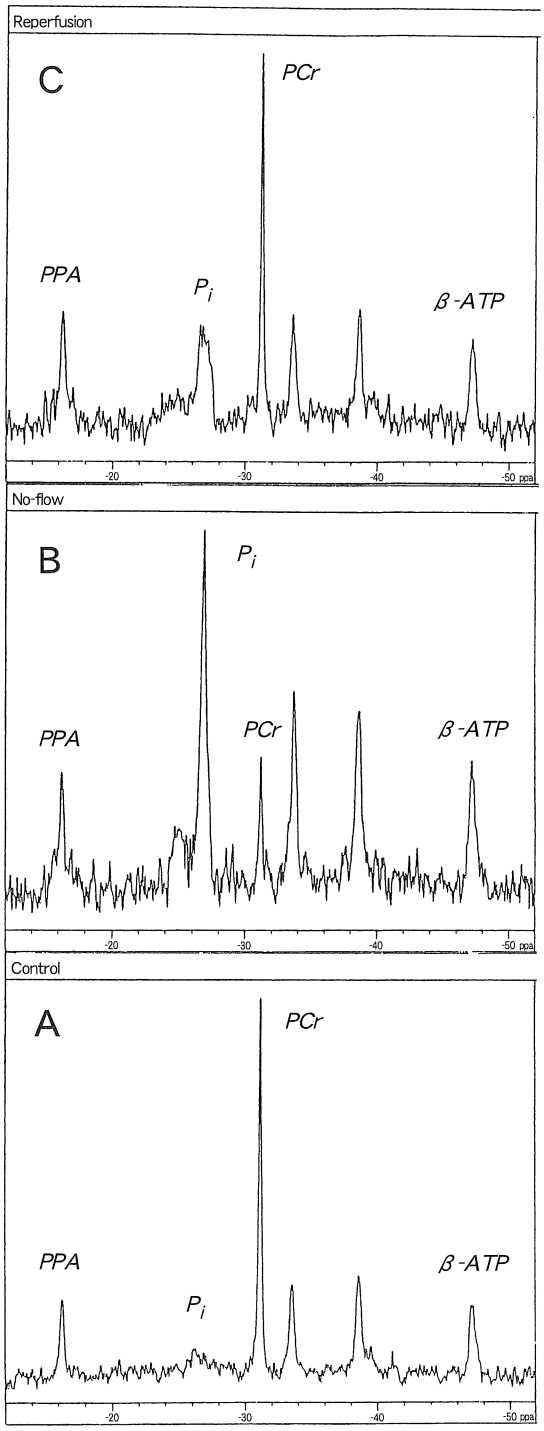

Fig. 3 Typical example of ^{31}P-NMR spectra of the perfused rat biceps femoris. Panel A : control, B : no-flow ischemia for 30 min and C : reperfusion for 30 min.

in the peak areas of these metabolites was observed during the perfusion with PPA. Figure 3B shows a spectrum observed after the no-flow ischemia for 30 min. During the no-flow ischemia P_i increased to a significant extent and PCr decreased markedly. PCr and P_i were recovered during the reperfusion period (Fig. 3C). These spectra were obtained from FIDs accumulated for 5 min each, and normalized with the respective largest peak ; PCr in the spectra of the control and the reperfusion, and P_i in the spectrum of the no-flow ischemia.

Metabolite concentrations and pH

The absolute values of the metabolites were obtained from the respective peak area of the MRS spectra by comparing with that of the PPA peak. A volume ratio of the muscle to the solution in the NMR tube was responsible to the determination of the absolute values. pH_i and pH_e were calculated from the chemical shifts according to Eqs. (1) and (2), respectively. Metabolites concentrations and pH during control, no-flow ischemia and reperfusion periods are summarized in Table 1. PCr decreased and P_i increased significantly by the no-flow ischemia and the both metabolites were recovered to the control levels by the reperfusion. ATP was not reduced even in the ischemic period. pH_i was significantly decreased by the no-flow ischemia and recovered to the control values by the reperfusion. On the other hand, pH_e was nearly constant throughout the experiments. The estimated pH_e values from Eq. (2) were found to be equivalent to the perfusate pH measured with a blood gas analyzer (Radiometer, ABL-50).

Time course changes of the metabolite and pH

Figure 4 shows the changes in metabolite concentrations, pH_i and pH_e during the control, no-flow ischemia and reperfusion periods. Date are shown as the mean and SD values for 6 rats. pH_i did not show a significant decrease until 20 min from the beginning of the no-flow ischemia. On the other hand, PCr showed a significant reduction at 10 min from the onset of ischemia. When PCr was reduced to about a half of the control level, pH_i began to show a significant fall. P_i showed significant increase with taking a mirror image of the PCr decrease. ATP and pH_e remained almost unchanged throughout the experiments. Figure 5 shows the rate constants of the changes in pH_i, PCr, P_i, ATP and pH_e during the no-flow ischemia by assuming exponential changes according to Meyer (1988). The data are shown as the

Table 1 Metabolite concentrations and pH of the pertused rat biceps femoris during control, no-flow ischemia and reperfusion periods.

Condition	PCr (mM)	ATP (mM)	P_i (mM)	pH_i	pH_e
Control	14.7 ± 2.14	6.43 ± 1.37	1.97 ± 0.52	7.07 ± 0.09	7.33 ± 0.06
No-flow (30min)	6.09 ± 1.04*	5.84 ± 1.25	8.92 ± 2.51*	6.87 ± 0.07*	7.28 ± 0.04
Reperfusion (30min)	13.5 ± 2.41	5.11 ± 1.01	2.02 ± 1.06	7.03 ± 0.15	7.34 ± 0.08

PCr, phosphocreatine ; ATP, adenosine-5'-phosphate ; P_i, inorganic phosphate ; pH_i, intracellular pH ; pH_e, extracellular pH. Data are means ± SD (n=6).
*P<0.05 compared with the control by the ANOVA test.

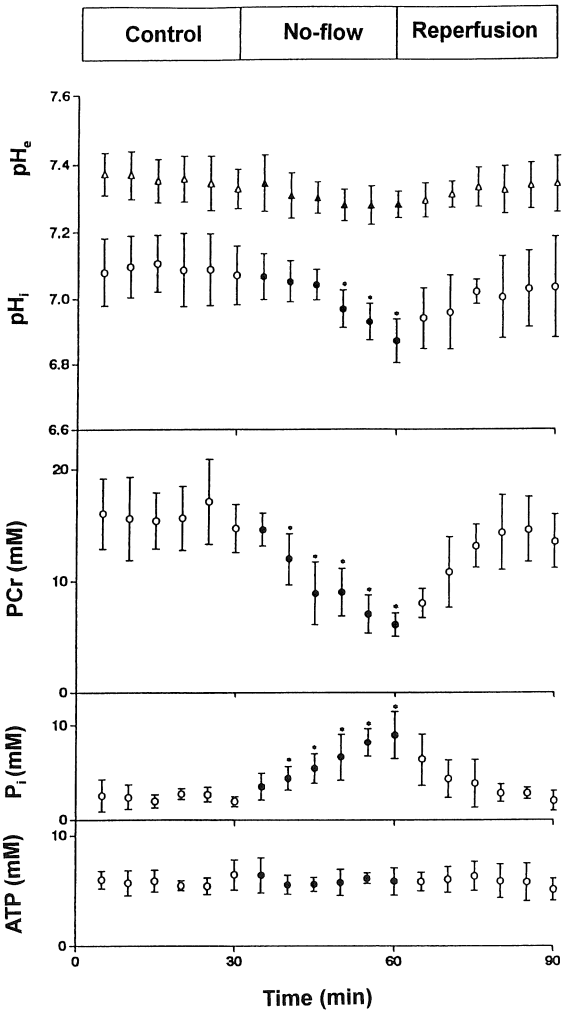

Fig. 4 Time course changes of pH_e, pH_i, and concentrations of metabolites (PCr, P_i and ATP) during the control, no-flow ischemia and reperfusion periods. (○ , △) ; data during the control and reperfusion periods, (● , ▲) ; data during the no-flow ischemia. Values are means ± SD (n=6) ; *P<0.05 compared with the control by the ANOVA test.

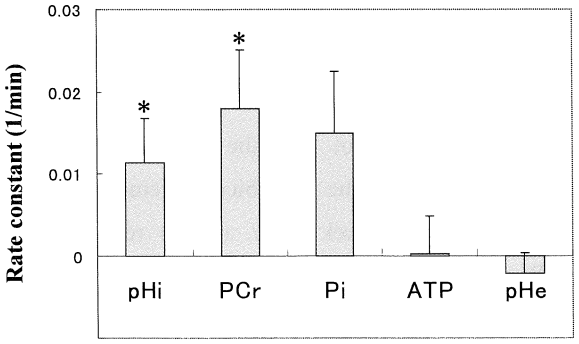

Fig. 5 Rate constants of changes in the metabolites concentrations, pH_i and pH_e during no-flow ischemia. Values are means ± SD (n=12) ; * P<0.05 by the ANOVA test.

mean and SD values of the time constants for 12 rats. The number of the rats for Fig. 5 were twice as that for Fig. 4, because we did some experiments without reperfusion for the determination of the rate constants during ischemia. In order to obtain the time constants of the exponential decay, pH_i and pH_e were converted to the respective hydrogen ion concentrations. The rate constant of PCr was significantly larger than that of pH_i, while there was no significant difference between the rate constants of PCr and P_i with the ANOVA test. The rate constants of ATP and pH_e changes were essentially zero.

DISCUSSION

Arterial perfusion at the body temperature used in this study seems to be an ideal preparation for the study of energy metabolism of skeletal muscle with ^{31}P-MRS. We measured PCr, ATP, P_i , pH_i and pH_e of the rat biceps femoris during the control, no-flow ischemia and reperfusion periods with perfusion ^{31}P-MRS.

As shown in Fig. 3, PCr decreased, P_i increased, and pH_i was reduced in the rat biceps femoris during ischemia. These changes in skeletal muscle are well accepted, but relations between the rates of changes in

the metabolite concentrations and pH_i are still unsolved. Highly-contained PCr in skeletal muscle may contribute to the buffering action of the pH_i decrease during ischemia. We planned to study energy metabolism of fast-twitch muscle because of the high PCr content in the muscle. Rat biceps femoris is one of the typical fast-twitch muscles (Ariano *et al.*, 1973). The control values of PCr, ATP and P_i of the rat biceps femoris determined were 14.7, 6.43 and 1.97 mM, respectively (Table1). The content of the fast glycolytic (type IIB) fiber in the rat biceps femoris is 49％ and that of the fast oxidative glycolytic (type IIA) fiber is 47％ (Ariano *et al.*, 1973). We could not find the reported metabolite concentrations of the rat biceps femoris in the literatures. Kushmerick *et al.* (1992) reported PCr, ATP and P_i of the rat extensor digitorum longus muscle to be 22.7, 6.7 and 1.1 mmol / kg, respectively, from their HPLC analysis. The unit mmol/kg approximately corresponds to mM (mmol / L). The rat extensor digitorum longus consists of 85％ fast glycolytic and 13％ fast oxidative glycolytic fibers (Kushmerick *et al.*, 1992). The lower content of PCr in the biceps femoris than in the extensor digitorum longus muscle is probably due to the lower content of the fast glycolytic fiber. ATP and P_i contents of the biceps femoris were nearly the same as those of the extensor digitorum longus muscle. A large size and shape of the muscle in the 25mm NMR tube enabled us to detect sufficient of phosphorus signals and to get reliable absolute metabolite concentrations.

ATP during ischemia can be supplied by creatine-kinase catalyzed reaction (Lohmann's reaction) :

$$PCr^{2-} + MgADP^{-} + H^{+} \rightarrow MgATP^{2-} + Cr, \quad (3)$$

where Cr is creatine. Lohmann's reaction is essential for stabilization and replenishment of the ATP pool at the expense of PCr (Wallimann *et al.*, 1992 ; Zelenznikar et al., 1995 ; Roman *et al.*, 1996). According to this reaction, PCr consumes H^+ to produce ATP. If ATP is provided by Lohmann's reaction, H^+ will decrease (Tanokura and Yamada, 1984 ; Roussel *et al.*, 2000). Another anaerobic ATP production is the glycolysis :

$$1/2Glu + P_i^{2-} + MgADP^{-} \rightarrow LA^{-} + MgATP^{2-} + H_2O, \quad (4)$$

where Glu is glucose and LA^- is lactate ion. ATP generation due to the adenylate kinase-catalyzed reaction can be neglected unless the reaction (3) is depressed (Zelenznikar *et al.*, 1995). On the other hand, ATP is consumed according to the ATP hydrolysis reaction :

$$MgATP^{2-} + H_2O \rightarrow MgADP^{-} + P_i^{2-} + H^{+}. \quad (5)$$

As shown in Fig. 4, ATP was essentially constant throughout the experiment, that is to say, the reactions (3) and (5) are synchronized with the balance of ATP input from PCr and output to P_i (PCr → ATP → P_i) (Uchida, 1998). Actually the PCr decrease was essentially a mirror image with the P_i increase (Fig. 4), and no significant difference in the rate constants was observed between the PCr decrease and the P_i increase (Fig. 5).

In our experiments with perfused rat biceps femoris, pH_i did not fall at the beginning of ischemia with sufficient PCr (Fig. 4). It should be noted that H^+ itself does not appear in Eq. (4). The source of proton is not the glycolysis but the ATP hydrolysis. Equation (4) can be obtained by summing up the sequential reactions of the glycolysis (Kashiwaya *et al.*, 1994). Lohmann's reaction can remove H^+ originated from the ATP hydrolysis, while the glycolysis cannot. With decreasing PCr, the main reaction for the ATP production may be switched from Lohmann's reaction to the glycolysis. The rate constant of the PCr decrease was significantly larger than that of the pH_i decrease (Fig. 5), corresponding to the PCr decrease by the no-flow ischemia was faster than the pH_i decrease as shown in Fig. 4. When PCr was reduced to about a half of the control level, pH_i began to show a significant fall. The pH_i fall in skeletal muscle during ischemia is possibly due to an alteration of the ATP source from Lohmann's reaction to the glycolysis. This turning point is likely to occur when PCr is reduced to about half of the control value (Fig. 4), or when the ratio PCr / (PCr + P_i) becomes less than 0.5. Such a turning point of the ATP source was also reported to appear during exercise (Sunoo *et al.*, 1996).

In conclusion, a perfusion ^{31}P-MRS technique is

suitable for the simultaneous measurements of metabolite and pH_i in intact muscle during control, ischemia and reperfusion periods. Our perfusion MRS measurements showed that the rate constant of the pH_i decrease during ischemia was significantly lower than that of the PCr decrease in the rat biceps femoris. These results suggest that PCr has a role to buffer the pH_i decrease during ischemia and contributes to ischemic tolerance of the fast-twitch muscle.

ACKNOWLEDGEMENTS

We wish to thank Dr. Daisuke Ishigaki for his technical assistance. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science and Culture of Japan.

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Received : December 12, 2001.

Accepted : January 22, 2002.