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Original paper

Relation between in vivo and in vitro measurements of oxidative metabolism in human tibialis anterior muscle

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Abstract

The purpose of this study is to examine the relationships between in vivo ^{31}P magnetic resonance spectroscopy (MRS) and in vitro markers of oxidative capacity in human tibialis anterior (TA) muscle. Twelve healthy male volunteers participated after giving written informed consent. Following 30 second isometric dorsiflexion exercise (70% of maximal effort), TA muscle mitochondrial adenosin triphosphate (ATP) resynthesis was determined from the phosphocreatine (PCr) recovery time constant (Tc), the maximal rate of PCr recovery (PCr_{rate}) and apparent maximal rate of oxidative ATP synthesis ($Qmax$). Muscle fiber type composition (I, IIA and IIX), citrate synthetase (CS) activity, capillary density (CD) and capillary-to-fiber ratio (C/F ratio) were determined from a biopsy sample of TA. Mitochondrial ATP resynthesis markers significantly correlated with CS activity and CD, but only weakly with fiber type composition and C/F ratio. These results indicate close relationships between in vivo and in vitro measurements of oxidative metabolism in human TA muscle.

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Key words: magnetic resonance spectroscopy, muscle biopsy, creatine phosphate, capillary citrate synthetase

Introduction

Human tibialis anterior (TA) muscle is comprised of about 70% type I fibers in biopsy studies (Johnson et al., 1973, Jakobsson et al., 1990, Gregory et al., 2001, Ratkevicius and Quistorff, 2002, Porter et al., 2002,), whereas nonhuman mammalian TA is comprised of less than 10% type I fibers (Lexell et al., 1994). However, despite its fiber type composition, human TA muscle has lower oxidative enzyme activity than gastrocnemius, vastus lateralis and soleus muscles (Gregory et al., 2001). Thus, TA is a unique muscle in lower legs. Recently, there is increased interest in studying the ankle dorsiflexor because of their important functions in gait and balance (Wolfson et al., 1995). TA is also thought to be a useful model for the examination of aging since it is used for locomotion and little else (Kent-Braun and NG, 2000).

Although much knowledge has been gained from histochemical and biochemical analyses of biopsy samples and from ^{31}P magnetic resonance spectroscopy (^{31}P MRS) of exercising muscle, few studies have adequately compared these invasive with noninvasive markers of oxidative metabolism. In contrast to the biopsy approach that permits analysis of fiber size, fiber type composition and

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maximal in vitro activity of oxidative enzymes, ^{31}P MRS allows for actual in vivo study of mitochondrial function (ATP resynthesis) in contracting muscles.

Phosphocreatine (PCr) represents an intramuscular energy buffer that can be degraded at an extremely high rate. PCr consumed by exercise was resynthesized by oxidative mitochondrial function. A maximal rate of PCr recovery (PCr_{rate}) should represent the maximal rate of oxygen consumption (Mahler, 1985; Meyer, 1988). Thus, PCr_{rate} and time constant of PCr recovery (Tc) is thought to be an indicator of muscle oxidative capacity in vivo. Two researchers reported that significant correlations were obtained between PCr_{rate} and citrate synthetase (CS) activity (McCully et al., 1993) and between Tc and the percentage of type IIA fibers (Larson-Meyer et al., 2001) in the gastrocnemius muscle, respectively. However, no study was conducted to examine these relationships in TA muscle.

TA muscle possesses unique histological properties. Electromyographic studies (Grimby, 1984, Jakobsson et al., 1988) revealed that TA muscle was mainly used during locomotion. Therefore, it is worthwhile to examine relationships in vivo (^{31}P MRS) and in vitro (needle biopsy) measurements of oxidative metabolism in TA muscle. The purpose of the present study is to elucidate this.

Materials and Methods

Subjects. Twelve healthy men [age 42.8 ± 4.6 yr, height 179.9 ± 2.8 cm, weight 76.0 ± 4.8 kg (mean ± SE)] were recruited for this study. All subjects were free of metabolic, cardiovascular and musculoskeletal disease and were recreationally active, but they were neither athletes nor participating in a regular exercise regimen. Informed consent was obtained from all subjects before participation. This study was also approved by the Ethics Committee of Copenhagen and Frederiskberg in Denmark, and the Ethics Committee of Sapporo Medical University.

Experimental Design. Experiments were conducted at a room temperature of 23 - 25 ℃. Subjects rested for approximately 30 min in the sitting position before the start of the experiment. They performed isometric dorsiflexion exercise to determine PCr recovery rate during recovery period. Phosphorus metabolites (Pi, PCr and ATP) and pH changes were measured during the protocol. The measurement probe of ^{31}P MRS was placed on the mid-portion of the TA muscle. One week later, needle biopsies were obtained from the same portion of the TA muscle, where the measuring probes of ^{31}P MRS experiment had been placed.

Experimental Protocol. Subjects sat upright in a chair with a vertical back support. The knee and ankle of their right leg were at an angle of about 170° and 110° , respectively. The foot was strapped to a plastic pedal, which was in tight contact with a strain gauge, in a self-made ankle-flexion ergometer. Maximum volitional isometric contraction (MVC) was determined as the best of three maximum dorsiflexion. Isometric dorsiflexion exercise was performed for 30 sec at intensity of 70% MVC after 10 min rest. Visual feedback of tension level was provided to subjects during exercise. Recovery was monitored for 7.5 min. The measurement protocol is illustrated in Fig. 1.

^{31}P MRS measurement. A magnetic resonance spectrometer (Vivospec; Otsuka Electronics, Fort Collins, CO, USA) and an 80 cm long 2.9 T horizontal superconducting magnet with 26 cm clear bore (3.0 T/31 mm Bore NMR Magnet; Magnex Scientific, Abingdon, England) were used. An inductively driven surface coil (3.5 cm diameter) was positioned on the mid-portions of TA. Homogenity of the magnetic field was optimized by shimming on the proton signal. The ^{31}P spectra were obtained at 49.79 MHz by single-pulse excitations (65 μS corresponding to a 90° pulse) and were collected in 1024 data points over 205 ms with 5-s inter-pulse interval. Total 252 single FIDs were acquired in the protocol. The FIDs were exposed to 5 Hz exponential line broadening, phasing, Fourier transformation and baseline correction, applying a cubic spline procedure (Phillips and Roberts 1983). Afterwards, the resonances were fitted to a Lorenzian line shape using a least-squares method providing the peak areas of Pi, PCr and ATP. The peak areas were corrected for the appropriate saturation factors and muscle metabolite concentrations were calculated assuming that the area of the γ-ATP peak (average of 10 FIDs) represents muscle ATP at rest. It has been reported that ATP is equal to 5.19 and 6.16 mmol (kg wet wt)$^{-1}$ in a type I and type II fiber, respectively (Sant'Ana Pereira et al. 1996). These values for ATP concentrations and individual data on muscle fiber type composition were used to estimate the average muscle ATP at rest before exercise in each subject. Intracellular pH was calculated from the chemical shift difference

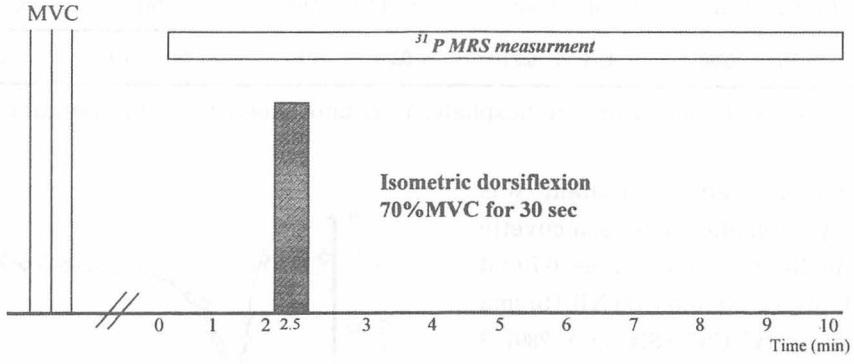

Fig. 1 Measurment protocol in this study.

between Pi and PCr (Arnold et al. 1984).

Mitochondrial ATP resynthesis. To estimate initial rate of PCr recovery, the ^{31}P MRS data were fitted to the following equation:

$$PCr_{(t)} = a \cdot (1-e^{-b \cdot t}) + c$$

where a is the drop in PCr, b is the rate constant, t is time and c is the difference between final and initial PCr concentration. The initial rate of PCr recovery (PCr_{rate}) is obtained as the derivative of equation in the point $t = 0$. The time constant of the PCr recovery (Tc) was also calculated by using the equation. Previous studies (Arnold et al. 1984, Taylor et al. 1986) demonstrated that PCr_{rate} was slower for intense than light or moderate exercise. This phenomenon has been attributed to both the effects of increased concentrations of hydrogen ion (i.e., decline of pH) on the creatine kinase equilibrium reaction, which shifts the equilibrium toward PCr hydrolysis, and the unavailability of substrate due to a depletion of the adenine nucleotide pool. ADP is also the controlling substrate for rate of oxidative activity (Chance et al. 1981). It appears therefore that the PCr_{rate} may be determined by other factors in addition to the rate of oxidative phosphorylation. Then, the PCr_{rate} is taken as the rate of mitochondrial ATP synthesis, assuming that by far the major part of ATP synthesis in this phase of recovery is used for PCr resynthesis. Then, PCr_{rate} was converted by Michaelis Menten kinetics and the actual free ADP concentration at the time point $t = 0$ calculated, assuming CK equilibrium. Km of oxidative phosphorylation for ADP is taken as 23 μM, allowing the calculation of $Qmax$ as follows:

$$Qmax = PCr_{rate} \cdot (Km + ADP) / ADP$$

Actual free ADP was calculated by an equation reported previously (Quistorff et al., 1993).

Muscle biopsy. Needle biopsies (Bergström 1962) were obtained from the middle portion of TA muscle, where the measuring probes ^{31}P MRS experiment had been placed. Local anesthetic (2 ml lidocaine, 1 %) was administered subcutaneously, and a small 1 cm incision was made in the skin and overlying fascia. One biopsy was obtained from each subject and divided into two pieces. Samples were immediately frozen in liquid nitrogen and stored at -80℃ until further histochemical and biochemical analysis.

Histochemical analysis. The first pieces were mounted in imbedding medium and frozen rapidly in isopenthane precooled with liquid nitrogen. Transverse serial sections (10 μm) were cut by a cryostat at -20℃ and stained for myofibrilar ATPase activity (Padykula and Herman, 1955) and with amylase-PAS (Andersen, 1975) staining to visualize capillaries. The sections were preincubated at a pH of 10.3, 4.6 and 4.3, and incubated for myofibrilar ATPase reaction at pH of 9.4. Fiber types were classified as three types, type I, IIA and IIX from a mean value of 273 (range 131−658) fibers in each biopsy. Muscle capillarization was expressed as capillary density (CD; the number of capillaries per one square millimeter) and capillary-to-fiber ratio (C/F ratio; total number of capillaries in a section divided by the number of fibers).

Biochemical analysis. The second pieces were divided into two parts and used for determination of citrate synthetase (CS) activity (Fink and Costill 1995). Small muscle pieces were weighted and homogenized in all-glass homogenizer in a 20 fold volume of a medium, 0.175 M KCL and 2 mM EDTA (pH 7.4). After a brief centrifuge (within 5 min) to

Table 1 ^{31}P MRS data collected during 2 min rest before exercise.

ATP(mmol·kg^{-1})	PCr(mmol·kg^{-1})	Pi(mmol·kg^{-1})	Pi/PCr ratio	pH	PCr/γ-ATP ratio
5.39 ± 0.03	17.76 ± 0.06	1.71 ± 0.21	0.09 ± 0.01	7.00 ± 0.01	3.44 ± 0.23

Values are mean ± SE. ATP; adenosine triphosphate, PCr; phosphocreatine, Pi; inorganic phosphate.

settle the cell debris, a clean supernatant was obtained. For spectrophotometic analysis, a cuvette was prepared individually in a 1 ml volume: 0.70 ml Tris buffer (100 mM, pH 8.3), 0.10 ml DTNB (Sigma D-8130, 1 mM), 0.15 ml Acctyl-CoA (Sigma A-2897, 3 mM) and 0.05 ml Oxalacerate (Sigma O-4126, 10 mM). Then, the cuvette was set into the spectrophotometer (UVmini-1240, Shimadzu Corporation, Japan) to 412 nm, zeroing on the reagent blank. Tissue homogenate of 10 μl was added into the cuvette and the change of absorbance was recorded every 15 sec for 4 min. CS activity [μmol · (kg wet w)$^{-1}$ · min^{-1}] was calculated by the following equation:

$$\text{CS activity} = abs/13.6 \cdot 2020$$

Where *abs* is the change of absorbance during 1 min, *13.6* is the molar extinction coefficient for DTNB at 412 nm and *2020* is dilution factors of muscle homogenate (20 times) and assay volume (101 times). All assays were run at a stable room temperature of 25 ℃.

Statistics. Values are presented as mean ± SE. A Pearson product-moment correlation coefficient was used to examine relationships between values. Results were considered statistically different at $p<0.05$.

Resutls

The ^{31}P MRS measurements were collected over a period of 2 min before exercise as shown in Table 1. During 70%MVC isometric exercise, actual tension level was 69.8 ± 0.70%. At the end of exercise, PCr decreased to 36.5 ± 5.9% of its resting value and pH dropped to 6.80 ± 0.06, respectively (Fig. 2). Tc, PCr_{rate} and *Qmax* are presented in Table 2. Muscle histochemical and biochemical properties are also shown in Table 3. Regression analysis denotes that ATP resynthesis markers were significantly correlated with CS activity and CD, respectively (Fig. 3 and 4), but not with fiber type composition (Table 4).

Discussion

Significant good correlations between three

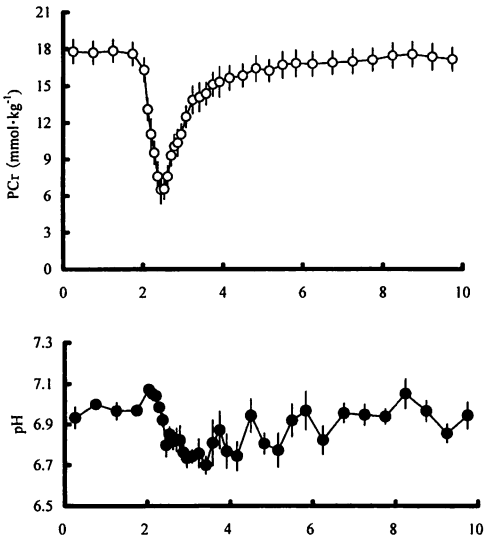

Fig. 2 PCr (upper) and pH (lower) changes during the protocol. Each X-axis represents time (min).

ATP resynthesis markers (Tc, PCr_{rate} and *Qmax*) and CS activity were found in this study. Up to this time, only two previous studies have compared ^{31}P MRS with biopsy techniques using ATP resynthesis markers and CS activity in human muscles (McCully et al., 1993, Larson-Meyer et al., 2001). In-magnet exercise consisted of plantar flexion exercise and needle biopsy samples were taken from the lateral head of the gastrocnemius in these studies. Larson-Meyer et al. (2001) showed that Tc following submaximal and maximal exercise was significantly correlated with CS activity ($r = -0.48$ and $r = -0.49$, respectively). McCully et al. (1993) also reported that the correlation between PCr_{rate} and CS activity had an r of 0.71. Larson-Meyer et al. (2001) indicated that *Qmax* following submaximal and maximal exercise was related with CS activity ($r = 0.63$ and $r = 0.64$, respectively). Our results are in agreement with two previous studies. Thus, we can accept close relationships between ATP resynthesis markers in vivo by ^{31}P MRS and CS activity in vitro by biopsy in skeletal muscles. As shown in Fig. 3, correlation coefficients of ATP resynthesis markers and CS activity are much higher in this study (Tc; −0.678, PCr_{rate}; 0.785,

Table 2 Mitochondrial ATP resynthesis markers.

Tc(sec)	PCr_{rate}(mmol·kg^{-1}·min^{-1})	*Qmax* (mmol·kg^{-1}·min^{-1})
32.3 ± 2.0	15.99 ± 1.49	21.75 ± 1.30

Values are mean ± SE. Tc; time costant of PCr recovery, PCr_{rate}; initial rate of PCr recovery, *Qmax* ; apparent maximal rate of oxidative ATP synthesis.

Table 3 Muscle histochemiacal and biochemical proprties.

% type I fibers	% type IIA fibers	% type IIX fibers	CS activity(μmol·kg^{-1}·min^{-1})	CD(capillaries/mm^2)	C/F ratio
74.8 ± 3.0	17.2 ± 2.2	8.0 ± 2.2	10.47 ± 0.77	301 ± 38	1.12 ± 0.17

Values are mean ± SE. %type I fibers; relative numbers of type I fibers, %type IIA fibers; relative numbers of type IIA fibers, %type IIX fibers; relative numbers of type IIX fibers, CS activity; citrate synthetase activity, CD; capillary density, C/F ratio; capillary to fiber ratio

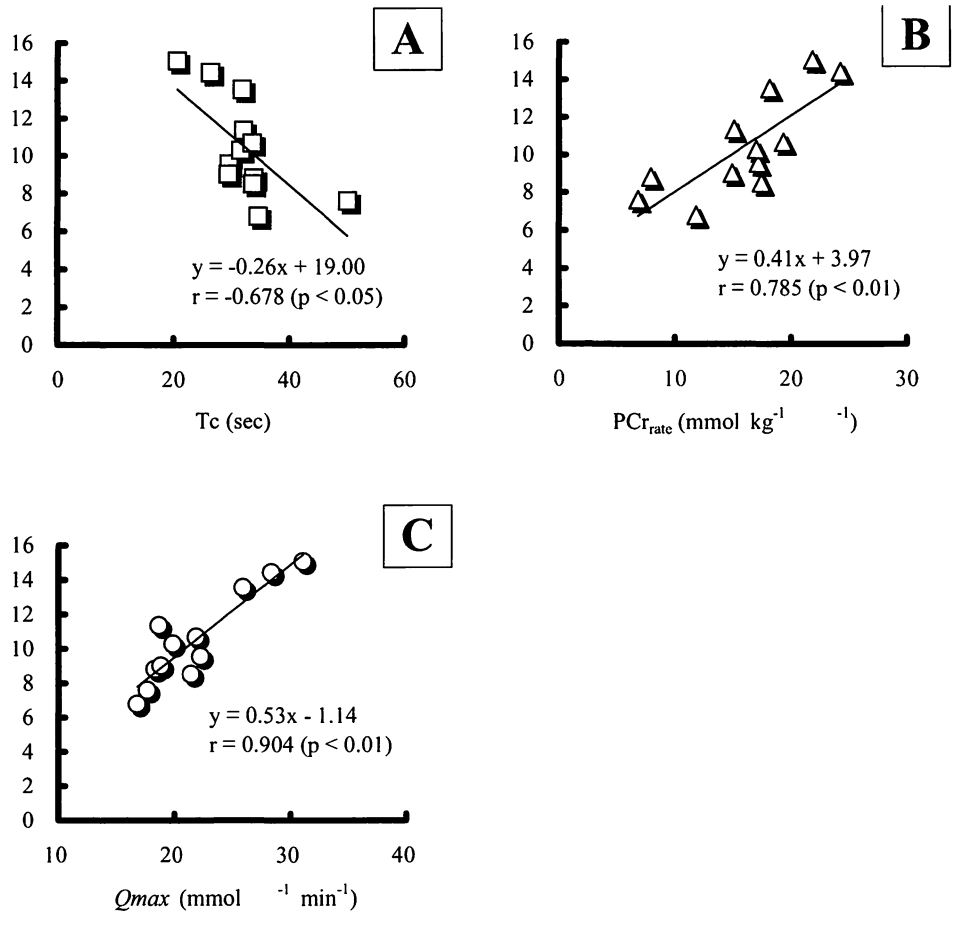

Fig. 3 Relationships between ATP resynthesis makers and CS activity. The panel A, B and C denote Tc, PCr_{rate} and *Qmax*, respectively. Each Y-axis represents CS activity (μmol·kg^{-1}·min^{-1}).

Qmax; 0.904) than the previous studies (McCully et al., 1993, Larson-Meyer et al., 2001). In planter flexion exercises, ^{31}P MRS data were acquired from the gastrocnemius-soleus complex and both muscles mainly work for exercise, but biopsy samples were obtained just from the gastrocnemius. In dorsiflexion exercise, TA is mainly a working muscle. Therefore, ^{31}P MRS and biopsy data in TA are closely correlated.

Tissue blood perfusion plays an important role of muscle oxidative metabolism (Andersen and Saltin, 1985). However, no study has examined relationships between ATP resynthesis markers and muscle capillary supply in spite of a strong

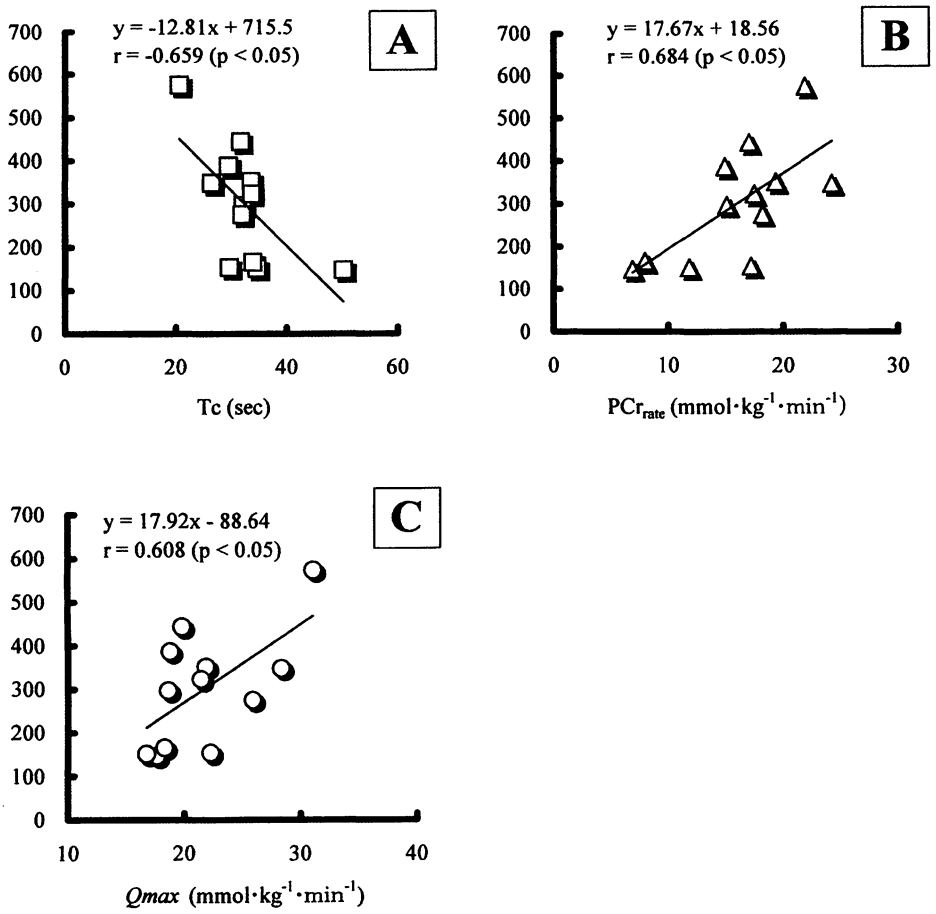

Fig. 4 Relationships between ATP resynthesis makers and CD. The panel A, B and C denote Tc, PCr_{rate} and *Qmax*, respectively. Each Y-axis represents CD (capillary numbers per mm^2).

Table 4 Correlation coefficients (r) between in vivo and in vitro measurments of oxidative metabolism.

	Tc	PCr_{rate}	*Qmax*
CS activity	−0.678	0.785	0.904
CD	−0.659	0.684	0.608
C/F ratio	−0.463	0.485	0.310
% type I fibers	−0.115	−0.122	−0.091
% type IIA fibers	−0.039	0.433	0.406
% type IIX fibers	−0.246	0.191	0.264

$r>0.576$; $p<0.05$, $r>0.708$; $p<0.01$, See abbreviation in Table 2 and 3.

relationship between Tc and tissue blood perfusion by a MRS-plethysmography study (Toussaint et al., 1996). To our knowledge, this is the first study to examine relations between ATP resynthesis markers and muscle capillary supply. As expected, we found significant moderate correlations between ATP resynthesis markers and CD (Fig. 4). This result shows that oxidative enzyme activity is not only important for ATP resynthesis but also muscle capillary supply, i.e., oxygen transport to muscle fibers. However, C/F ratio did not related with any ATP resynthesis markers. As morphometric indexes used to describe muscle capillary supply, CD and C/F ratio represent the number of capillaries per unit area (mm^2) and ratio of capillary density to fiber density, respectively. Therefore, "quantity" rather than "quality" in muscle capillarization may be an important factor for ATP resynthesis following exercise.

We found significant relationships between

ATP resynthesis markers, and CS activity and CD (Fig. 3 and 4), surprisingly but not fiber type composition (Table 4). Two studies (McCully et al., 1993, Larson-Meyer et al., 2001) examined relationships between ATP resynthesis markers and fiber type composition, but the findings are conflicting. McCulley et al. (1993) suggested no correlations with PCr_{rate} and muscle fiber composition of gastrocnemius. Conversely, in the same muscle, Larson-Meyer et al. (2001) demonstrated moderate correlations between ATP resynthesis markers (Tc and *Qmax*) and the percentage of type IIA fibers but not that of type I and type IIX fibers. Our result is in agreement with the former study. From the results of three studies, however, it may be the fact that the percentage of type I fibers does not contribute to ATP resynthesis following exercise.

Larson-Meyer et al. (2001) reported that the mean percentage of type IIA fibers is 30.4 ± 9.9% (16.5% − 50.7%) in their study. On the other hand, we found that type IIA fibers occupied 17.2 ± 2.2% (8.7% − 34.0%) when counted by number (Table 3). In addition, the number of subjects was smaller in our study (n=12) than that of Larson-Meyer's study (n=23). Homogeneity of distribution in type IIA fibers and smaller sample size may influence the lack of correlation between ATP resynthesis markers and % type IIA fibers in this study. Essentially, the percentage of type IIA fibers probably must contribute to ATP resynthesis after exercise because, as shown in Table 4, % type IIA fibers moderately related with PCr_{rate} and *Qmax* in our data (but not significant), and its correlation coefficients were the highest in three fiber types. The reason for the discrepancy in fiber type composition among the studies is not clear. One possible reason is that sampling from three to five sites maybe necessary to characterize adequately a whole muscle (Elder et al., 1982, Coyle et al., 1992).

Conclusion

In human tibialis anterior muscle, the primary ankle dorsiflexor, we found close relationships between in vivo and in vitro measurements of oxidative metabolism. In particular, ATP resynthesis markers following exercise were correlated with mitochondrial oxidative enzyme activity and capillarization. Our experimental model should serve to advance the use of ^{31}P MRS in future research directed at determining mitochondrial oxidative capacity in vivo.

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