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Improved high-performance liquid chromatographic assay for the determination of "high-energy" phosphates in mammalian skeletal muscle
Application to a single-fibre study in man

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Abstract

A sensitive and reproducible method for the determination of adenine nucleotides (ATP, IMP) and creatine compounds [creatine (Cr), phosphocreatine (PCr)] in freeze–dried single human muscle fibre fragments is presented. The method uses isocratic reversed-phase high-performance liquid chromatography of methanol extracts. Average retention times (min) of ATP, IMP and PCr, Cr from standard solutions were 10.6±0.42, 2.11±0.06 (n=6) and 10.5±0.31 and 1.19±0.02 (n=9), respectively. Detection limits in extracts from muscle fibre fragments were 2.0, 1.0, 3.0 and 2.0 mmol/kg dm, respectively. The assay was found successful for analysis of fibre-fragments weighing ≥1 μg. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Phosphocreatine; Creatine; Adenosine triphosphate; Inosine monophosphate

1. Introduction

Many laboratories are interested in the quantification of high-energy phosphates and related compounds in mammalian skeletal muscle. The determination of phosphocreatine (PCr), creatine (Cr), adenosine triphosphate (ATP) and inosine monophosphate (IMP) levels in biopsy samples is of importance for the examination of the biochemical bases of human muscle function in normal and pathological situations and it is frequently performed in studies of exercise metabolism. During brief high-intensity muscle contraction PCr is rapidly dephosphorylated to Cr and reductions in ATP levels are accompanied with stoichiometric increases in IMP and ammonia while absolute ADP and adenosine phosphate (AMP) levels are only slightly increased [1,2].

Most frequently whole muscle extracts have been analysed using methods that include separate enzymatic assays [3] and high-performance liquid chromatography (HPLC) [4–6]. Also combinations of different methods on whole muscle and single fibres [7] and on fibre pools [8,9] have been per-

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formed. At the single fibre level, enzymatic cycling has been used for determination of ATP and PCr in rat [10] and human [11] and HPLC for ATP and IMP separation in human muscle fibres [12]. A bioluminometric method has also been described for the determination of ATP and PCr in single human muscle fibres [13].

The enzymatic cycling assays involve several solutions and several stages of analysis. The reported bioluminometric method is less complicated but it does not measure Cr and IMP levels. Moreover the above mentioned HPLC methods used perchloric acid extraction which has been shown to interfere with the retention behaviour of nucleotides [14]. Generally, acid extraction involves neutralisation procedures and consequently more steps in sample preparation, which may affect accuracy and reliability of the results.

For the purposes of our group's research we wanted to have determination of ATP, IMP, PCr and Cr from the same muscle fibre extract, something which was not accomplished by any of the previous mentioned methods, while still using isocratic analysis. High sensitivity was needed in order to be able to detect any variation in changes across the population of different fibre types found in skeletal muscle. We also wanted to employ the simplest extraction procedure possible.

The present paper describes an improved reverse-phase isocratic HPLC assay for determination of high-energy phosphates in methanol extracts from fragments of single human muscle fibres.

2. Experimental

2.1. Chemicals

All chemicals were of highest degree of purity. ATP, IMP, PCr, Cr and tetrabutylammonium hydrogensulphate (TBAHS) were obtained from Sigma (St. Louis, MO, USA). HPLC-grade methanol, acetonitrile and potassium dihydrogenorthophosphate (KH_2PO_4) were purchased from Merck (Darmstadt, Germany). Stock solutions were stored in Eppendorf vials at −20°C. Only double-distilled deionized water (Labconco, Water Pro PS, Beun de Ronde, The Netherlands) was used and working solutions were prepared fresh before each run.

2.2. Instrumentation

The HPLC system consisted of a Binary LC pump (Model 250, Perkin-Elmer, USA), an autosampler with cooling tray and automatic injector (Basic Marathon, Spark Holland, The Netherlands) and a variable-wavelength ultraviolet (UV) spectrophotometric detector (Model 759A, Applied Biosystems, The Netherlands). The size of the injection loop was 20 μl. For ATP–IMP and PCr–Cr separations the UV absorption was measured at 254 and 210 nm, respectively. Peaks were identified and quantified by a Chromatography Data System (Model 717, Axxiom Chromatography, CA, USA) by comparing sample peak heights to those of external standards.

2.3. Procedure

Analysis was carried out at controlled room temperature (20°C), under isocratic conditions using a reversed-phase 125×4 mm analytical column protected by a 4×4 mm guard cartridge (both 5 μm particle size, RP-18 LiChrosphere 100, Hewlett-Packard, The Netherlands). The mobile phase was pumped at a flow-rate of 1 ml/min. For ATP and IMP analysis the mobile phase (A) consisted of a 215 mmol/l KH_2PO_4, 2.3 mmol/l TBAHS and 2% acetonitrile aqueous solution adjusted to pH 6.5 with 5 *M* KOH. For PCr and Cr analysis the mobile phase (B) consisted of 14.7 mmol/l KH_2PO_4, 1.15 mmol/l TBAHS aqueous solution adjusted to pH 5.3 with 5 *M* KOH. The eluents were degassed before use and were kept under helium during analysis. Before an assay the column was washed and equilibrated with the mobile phase.

2.4. Standard solutions

For the calculation of the concentrations of ATP, IMP, PCr and Cr in tissue samples, standard solutions of these components were used. Two stock solutions were prepared in 215 mmol/l potassium (pH 7.0). Stock A contained 0.393 mmol/l ATP, 0.114 mmol/l ADP, 0.072 mmol/l AMP and 0.527 mmol/l IMP. Stock B contained 1.61 mmol/l Cr and

1.50 mmol/l PCr. Based on the dilution during extraction of muscle samples (explained later) and the ranges of metabolite concentrations reported in the literature for mixed muscle [15,7,21], single fibre pools [17,9] and single fibres [12,16,18–20], we estimated the expected final concentrations in the muscle samples and used these to set the range of our standards' concentrations (Table 1).

Calibration curves were initially obtained by injection of standard solutions using nine different concentrations per compound. As the resulting linear regression analysis of these calibrations curves was excellent and retention times very stable (data not shown), we decided to limit ourselves to a conventional four-point calibration. Concentrations were prepared by diluting: stock solution A either 200-, 1000-, 4000- or 8000-fold with mobile phase A and stock solution B either 1600-, 4000-, 8000- or 16 000-fold with mobile phase B. Calibration data were plotted and linear regression lines were calculated using the least-squares method. For routine analysis of tissue extracts standards of different dilutions were injected regularly, one every four samples.

Samples' concentrations were calculated from comparison of the peak heights to those of the calibration standards. Peak heights were used rather than peak area, because previous data showed that the variability of repeated measures was lower for peak height measurements [14].

2.5. Sample preparation

2.5.1. Animal muscle

Medial gastrocnemius (GM) muscles were obtained from Wistar rats, which had their leg muscles electrically stimulated (one leg exercised, the other control). The stimulation was performed as a part of another study. The muscles were freeze–clamped and stored in liquid nitrogen. On a later occasion some muscle samples were manually powdered under nitrogen and freeze–dried (see e.g., de Haan et al. [22]) while others were dissected to provide fibre bundles.

Table 1
Range of concentrations of metabolites of interest in the literature; in our estimates; and in our standards

Metabolite	Literature (mmol/g dm)	Estimated (mmol/l)	Standard (mmol/l)
ATP	5.00–30.0	0.04–0.20	0.05–1.95
IMP	0.00–20.0	0.00–0.16	0.06–2.64
PCr	6.00–90.0	0.05–0.50	0.09–0.94
Cr	40.0–120	0.20–0.70	0.10–1.00

2.5.2. Human muscle

Biopsy samples of the quadriceps femoris muscle were obtained, using a Bergstrom-type biopsy needle with suction, from two healthy male volunteers as part of a larger study, which had ethics committee approval. From both individuals we obtained samples in the resting and fatigued state (immediately after 25 s high-intensity cycling). Samples were instantly frozen in liquid nitrogen and freeze–dried overnight. Single fibres were dissected under conditions of controlled temperature and relative humidity (21°C and 25%, respectively) and parts of the single fibres were used to characterise the fibre type histochemically, basically as described by Sant'Ana Pereira et al. [9].

Methanol (60%, v/v) [23] was added to the remaining fraction of the single fibres, pools of fibres or powdered muscle tissue and mixed. Extraction of metabolites occurred overnight at −80°C. For rat muscle powder and human muscle fibre pools, 1 μl of 60% methanol was added per μg of tissue. For human single muscle fibres 20 μl was the amount added irrespective of the fibres' mass. Preliminary work did not identify any significant loss of metabolites from muscle after prolonged storage at −80°C before or after extraction (data not shown).

The samples were mixed, centrifuged (12 000 *g* at 4°C for 20 min; Biofuge 22R, Heraeus Sepatech, Germany) and the supernatants were separated in two portions: two thirds for ATP–IMP and one third for PCr–Cr analysis. Subsequently, the samples were carefully dried under nitrogen gas, while on ice, and either stored at −80°C until further analysis or immediately dissolved in 90 μl of their respective eluents and analysed on the day.

2.6. Repeatability and consistency

Between-day repeatability of retention times was assessed by the analysis of standard A on six different days and of standard B on nine different

days. Within-day repeatability was assessed by monitoring the values of initial calibration standards and standards analysed during the actual experimental assay. Consistency in the analysis of the chosen metabolites between repeated runs of the same samples was assessed by dividing rat leg powdered muscle in two portions and analysing both for ATP–IMP and PCr–Cr contents. The precision of the method for real muscle samples was further assessed by analysing four different extractions of different portions (0.05, 0.10, 50 and 100 mg) of powdered resting rat GM muscle on four different occasions. Moreover, fibre bundles from resting rat GM muscle were cut to a range of masses (6–50 μg) and analysed on four different occasions.

2.7. Detection and quantification limits

With a further dilution of standard solutions, the lowest detectable concentrations (signal-to-noise ratio 2:1) were obtained for all metabolites (detection limits). Using these detection limits, the lowest quantifiable concentrations in a 1-μg fibre fragment were calculated (quantification limits).

2.8. Recovery of metabolites

The percentage recovery of ATP, PCr and Cr was examined using the standard addition method. Standards were added to 12 fragments (25 to 45 μg dm) belonging to four different fibre bundles of rat GM. Analysis was performed on three fragments per bundle, which were alternated with corresponding fragments of identical mass but where standards were not added.

2.9. Human single fibres

Values obtained from pools of type I human single fibres were compared with the values obtained when analysing individual single fibres of the same type. Mean TCr of individual human single fibres was compared between samples from the fatigued state and those from the resting state and against TCr from fibre pools.

2.10. Statistics

Standard descriptive statistics were used to present data as mean value±standard deviation (SD). Relative standard deviations (RSDs) were calculated (standard deviation×100/mean) for assessing repeatability of retention times from standards analysed on different occasions. Intra-class correlation (R), which uses analysis of variance (ANOVA) to obtain the reliability coefficient (ranging from 0 to 1), was calculated as an estimate of reliability and consistency of the analytical method (we accepted any trial-to-trial variation as measurement error, as proposed by Thomas and Nelson [25]). The t-test was employed for assessing the duplicate analysis of rat leg muscle. Correlation coefficient (r) calculations were used to demonstrate any relationships between compounds. All statistical analyses were performed at a significance level of $P<0.05$ using a commercially available statistical package (SPSS 6.0 for Windows, SPSS 1993).

3. Results

The method we report is based on previous work from Sellevold et al. [26]. They used one assay for both adenine nucleotides and creatine compounds by compromising the detection wavelength. Initially we attempted to employ a single run. However, in our skeletal muscle samples the creatine peak was poorly resolved from the first injection peak and all metabolites were eluted very closely making identification of the peaks difficult (also noted by Sellevold et al. [26]). Because we considered it important to have the total creatine content of our single fibre extracts, we decided to use two separate runs on the same extract; peaks were detected at the respective optimal wavelength for creatine compounds and adenine nucleotides.

Muscle metabolites are expressed as mmol per kg dry muscle mass (mmol/kg dm).

3.1. Chromatography of standards

Calibration curves were obtained for ATP, IMP, PCr and Cr. Least-squares regression lines for all metabolites had intercepts near zero with correlation

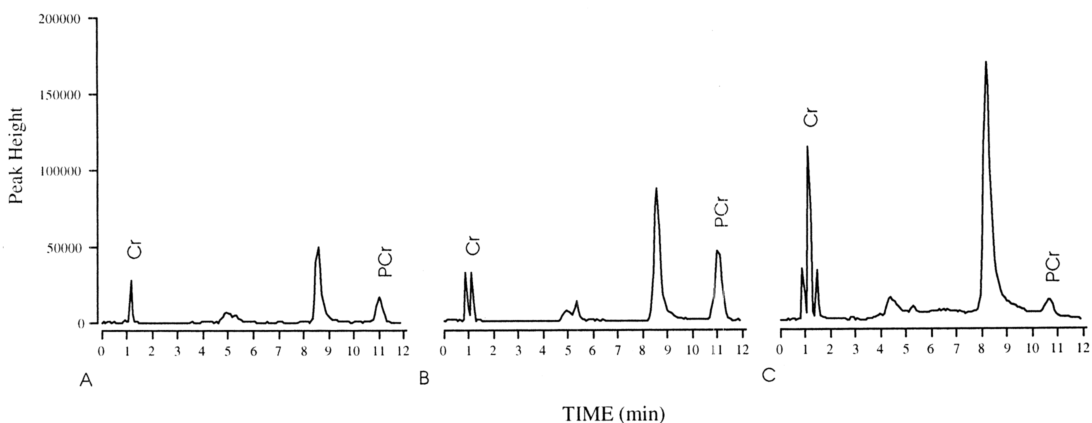

Fig. 1. Separation of PCr and Cr by isocratic reversed-phase HPLC. Typical chromatograms are shown for standard solution (A), resting type II fibre (B) and post-exercise type II fibre (C). For elution conditions see Experimental.

coefficients higher than 0.999. Typical examples of standard chromatograms are presented in Figs. 1A and 2A. Repeatability of measurements in different occasions was assessed by calculating the RSD of the calibration slopes of the different analytical days (Table 2). Also the RSD values of between-day analysis of the highest dilution standard are presented (Table 2).

Within the same day analysis, retention time of the compounds of interest hardly showed any variation, irrespective of standard concentration (all dilutions were tested, data not shown). The average of the day

Table 2
Repeatability of measurements of nucleotides and creatine compounds in standards

Standard	RSD (%)[a]	
	Slope (*n*)	Peak height (*n*)
ATP	4.3 (5)	15.6 (5)
IMP	3.8 (5)	11.2 (5)
PCr	5.2 (11)	10.6 (4)
Cr	4.2 (11)	7.2 (4)

[a] Relative standard deviations (RSDs, %) are shown of the slopes of the regression lines using all standard dilutions, and of the peak height at the highest dilution (n=number of runs).

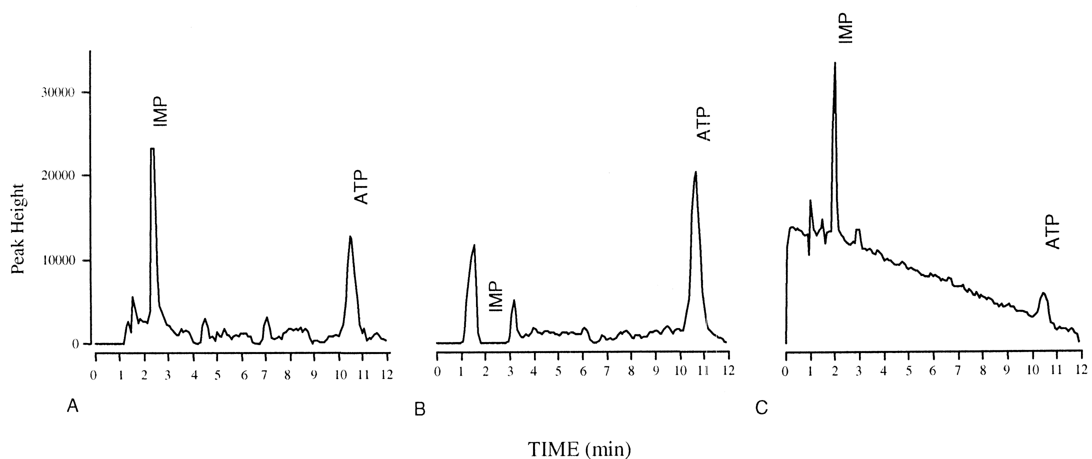

Fig. 2. Separation of ATP and IMP by isocratic reversed-phase HPLC. Typical chromatograms are shown for a standard solution (A), resting type I fibre (B) and post-exercise type II fibre (C). Note that in some post-exercise samples the baseline was shifted as shown here in panel C. For elution conditions see Experimental.

was taken and compared on different occasions. The repeatability of the analysis over time is demonstrated by the small variation observed in the retention times of each of the compounds of interest (Table 3). We attribute the small variations in retention times to the natural wearing of the column as well as inevitable small variations in the eluents' composition. For the same data, the reliability coefficient for both standards A and B was $R=1$.

3.2. Recovery of metabolites

Recovery of ATP, PCr and Cr was examined using the standard addition method on fragments of rat GM fibre bundles. This aimed at examining the recovery curves for the extraction procedures, which showed recovery of 94.2±0.9% for ATP, 89.6±1.2% for PCr and 92.3±1.4% for Cr ($n=12$ for all metabolites). Because the recovery was relatively high, it was decided that no correction of data was needed.

3.3. Chromatography of muscle samples

3.3.1. Animal muscle

The precision of the analytical method for muscle samples was initially assessed by repeated analysis of four different muscle extracts of resting rat GM muscle. The RSDs for the repeated measures of different muscle extracts (in total $n=16$) were 3.20% for ATP, 2.18% for PCr and 2.96% for Cr. The IMP concentration of these resting muscle extracts was too low to quantify. Similarly, RSD values for the resting GM fibre bundles analysed were 7.08%, 3.00% and 2.99% for ATP, PCr and Cr, respectively. Furthermore, seven exercised powdered rat gastrocnemius muscle samples were divided in two parts and were analysed for ATP–IMP and PCr–Cr levels. The concentration range was 21–44 for ATP, 0.2–5.5 for IMP, 33–130 for PCr and 103–205 for Cr, values in mmol/kg dm. No significant differences were found between the two sets of analysis (t-test). The percentage agreement between the duplicate measurements was 99.5±7.9% for ATP, 98 ±19% for IMP, 98.5±6.2% for PCr and 100±2% for Cr levels. When plotting all IMP vs. ATP values and all PCr vs. Cr values, correlation coefficients (r) of −0.896 and −1.00, respectively were obtained ($P<0.05$), these inverse strong relationships verifying the validity of our results.

3.3.2. Human single fibres

Two muscle biopsies were collected, one at rest and one immediately after 25 s maximal cycling exercise from two subjects (X and Y) with different activity background. Single fibres were dissected and histochemically characterised and analysis was performed both on pools of type I and type II fibres (25.3–59.4 μg) and on individual single fibres. In our experience, fibre fragments of less than 1 μg mass (~1 mm length) could not be analysed reliably so we processed only fibre fragments with a mass of more than 1 μg. Characteristic chromatograms of single fibre extractions are presented in Figs. 1B and C and 2B and C. Quantification limits for the different metabolites in 1 μg of fibre tissue are presented in Table 4.

One pool of type I fibres from the two biopsies of subject X had a TCr content of 102 mmol/kg dm. Individually analysed type I fibres (1.0–3.3 μg) from resting and post-exercise samples had a mean TCr of 120±20 ($n=4$) and 107±10 mmol/kg dm ($n=6$), respectively. Single type II fibres (1.4–3.2 μg) had a TCr of 120±30 and 120±34 mmol/kg dm at rest ($n=14$) and post-exercise ($n=8$) respectively. The

Table 3
Retention times for nucleotides in standard solution A at six different occasions and for PCr–Cr in standard solution B on nine different occasions (values are mean±SD and RSD, %)

Compound	Retention time (min)	RSD (%)
IMP	2.11±0.06	2.8
AMP	4.09±0.14	3.5
ADP	6.73±0.18	2.8
ATP	10.6±0.42	4.0
PCr	10.5±0.31	2.9
Cr	1.19±0.02	2.1

Table 4
Quantification limits for ATP, IMP, PCr and Cr (values are expressed both as mmol per litre and as mmol per kilogram dry mass)

Compound	mmol/l	mmol/kg dm
ATP	0.02	2.00
IMP	0.01	1.00
PCr	0.02	3.00
Cr	0.01	2.00

Table 5
Nucleotide and creatine compounds concentrations (mmol/kg dm) in human single muscle fibres (values are mean±SD, n=number of single fibres analysed)

	ATP	IMP	PCr	Cr
Subject X				
Type II				
Rest (n=14)	20±7	n.q.[a]	66.4±6.2	54±6.2
Post-exercise (n=10)	8±4	11.1±0.9	15±8	105±8
Type I				
Rest (n=4)	10±3	n.q.	62.1±3.0	37.9±3.0
Post-exercise (n=6)	10±4	3±2	10±6	88.6±5.7
Subject Y				
Type II				
Rest (n=7)	22±3	n.q.	117±3	52.7±2.9
Post-exercise (n=5)	6±2	23.4±1.6	10±6	159±6
Type I				
Rest (n=13)	20±4	n.q.	95.3±4.3	44.7±4.3
Post-exercise (n=7)	15±2	10±7	25±5	115±6

[a] n.q.=Under quantification limit.

type II fibres analysed were mainly of type IIA. Similar differences between type I and II fibres were found for subject Y. The difference between subjects X and Y was in terms of TCr. For subject Y, resting TCr of type I fibres' pool was 140 mmol/kg dm while for type II fibres pool resting TCr was 170 mmol/kg dm.

Normalisation of nucleotide and creatine content of muscle in exercise protocols based on TCr is a common practice (e.g., [15,16,24,27]). The observed differences in TCr between type I and II fibres prompted us to consider normalisation of single fibre values with the data of the average TCr pool for the corresponding fibre type (e.g., [single fibre PCr value×fibre pool TCr]/[single fibre TCr]). So for subject X, type I and type II fibre values from resting as well as from post-exercise samples were normalised for TCr of 100 and 120 mmol/kg dm, respectively. Similarly for subject Y, type I and type II fibre values were normalised for TCr of 140 and 170 mmol/kg dm, respectively. Detailed results are presented in Table 5.

4. Discussion

The aim of the present study was to obtain a reliable method for measuring ATP, IMP, PCr and Cr in single human skeletal muscle fibre fragments. Our results indicate that the method developed is reproducible, accurate and sensitive enough for our objective, allowing for the first time both nucleotide and creatine compounds separation to be performed in single muscle fibres.

4.1. Method development observations

The method was optimised to obtain a good separation of metabolites with sharp peaks by systematic variation of the mobile phase qualities (A and B) when analysing standard solutions. For PCr–Cr analysis, using TBAHS concentrations higher than 1.15 mmol/l in mobile phase B, lead to shorter retention times, sharper peaks, and higher peak heights for PCr, but compromised the separation of the Cr peak. Lower concentrations of TBAHS resulted in higher retention times and lower peak heights. Lowering the pH of mobile phase B below 5.3 made separation of the Cr peak impossible. Increasing pH above 5.3 not only affected peak height, but also the separation of the PCr peak from the unidentified peak (see Fig. 1; retention time ~8.5 min). This unidentified peak varied in peak height randomly between muscle samples, and its retention time varied differently with pH compared with the PCr peak. For mobile phase A, pH 6.5 was found to

be the best compromise between retention times and distinct separation of nucleotides. Addition of acetonitrile and a high salt concentration were necessary for short time elution of ATP (within 11 min). Wrapping the column with insulation material to "stabilise" its temperature for varying room temperatures (from 18 to 23°C) had no effect on reproducibility of the measurements. To maintain high performance of the system, the guard cartridge had to be replaced every 30 to 40 injections. Between 100 and 400 injections could be performed on the analytical column. This column was replaced when the peak height to area ratio was decreased by 5% compared with the situation in the new state.

4.2. Characterisation of the established HPLC assay

The stable retention times verify that optimal solutions were found for the separation of the metabolites of interest [26]. By mixing standard solutions to actual muscle extracts, the interference of tissue extracts on known quantities of the compounds of interest was evaluated. The recovery values showed that there was no interference of practical significance. Previously, Dunnett et al. [6] observed no co-elutants absorbing at 210 nm. Moreover, in a previous study, it was demonstrated that measurements of the relevant compounds with HPLC gave similar results as enzymatic analyses [14].

In the present study, values obtained from the human single fibres were normalised for the TCr content of corresponding fibre pools. Muscle TCr content is frequently used as a reference base for reporting intracellular metabolite content [21,24,27,28]. This allows to compensate for possible weighing errors or errors due to any differences in metabolites along the fibres length and due to any variability in biopsy composition arising from exercise hyperaemia [18,27].

4.3. Concentration of high-energy phosphates in single human muscle fibres

Our results agree with the existing literature in that little to no variation in TCr is observed pre- and post-exercise [7,8,28]. The TCr values of our two subjects occupy the two ends of the range reported by others [7,9,12,15–21]. The agreement in TCr content and PCr/Cr ratio of single fibres with that of respective pools of fibres adds credence to our method.

In spite of individual variations in TCr and PCr content, for both subjects the fatiguing protocol imposed maximal metabolic demands on their working muscles reflected by the decrease in PCr and increase in IMP levels (Table 5). The mean PCr/Cr ratio for type I fibres was reduced by ~92% and ~90% for subjects X and Y. For type II fibres the reductions were ~ 88% and ~ 96% for the respective subjects. Previous studies, after enzymatic analysis of mixed muscle, reported TCr content ranging from ~100 to ~140 mmol/kg dm and resting PCr/Cr ratios ranging from ~1.58 to ~2.1 [8,21,28,29]. The latter ratio was reduced to 0.27 (~87% reduction) after a 30 s sprint exercise [21]. Our results on TCr content and PCr/Cr ratio measured in single fibres are consistent with these previous reports on mixed muscle.

Earlier studies have reported ATP resting levels of 27.05±0.95 mmol/kg dm in a mixed muscle sample [29] and as low as 14 mmol/kg dm in single type I fibres [12], sampled from the vastus lateralis. Some researchers have reported significantly higher ATP content in type II than type I fibres [9,19] while some have been unable to detect significant differences [7,28]. In our study, we observed somewhat higher resting ATP content in type II vs. type I fibres, especially for subject X (Table 5). It was reported that after 25s isokinetic cycling reductions in ATP were greater in type II vs. type I with higher levels of IMP in type II fibres [9]. In our study greater reductions in ATP levels of type II vs. type I fibres were observed in both subjects accompanied with higher IMP levels for type II fibres (Table 5).

4.4. Limitations and concluding remarks

Some differences in resting metabolites observed within the same fibre types have been also noted by others (e.g., [28]) and can be mainly attributed to physiological variations of the high energy phosphate content of the fibre fragments rather than methodological problems. It should be noted that in other single fibre studies the available methods did not allow for the Cr or TCr content for single fibres to be

analysed, but only for mixed homogenates. Here we report individual fibre results from two subjects with different TCr, ATP content and PCr/Cr ratio, whose values occupy the two ends of the reported normal ranges. The fact that subject X was an endurance-trained athlete while subject Y was not specifically trained may account for their differences.

Based on our results, we can conclude that we have developed a sensitive method to measure ATP, IMP, PCr and Cr concentrations in human muscle single fibre fragments. This method allows us to quantify exercise-induced changes in these metabolites in single muscle fibres with different metabolic and contractile properties.

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