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Standardisation of ^{31}phosphorus-nuclear magnetic resonance spectroscopy determinations of high energy phosphates in humans

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Abstract. A procedure is described for standardising the determination of adenosine 5′-triphosphate and phosphocreatine concentration ([ATP] and [PC], respectively, in absolute arbitrary units) in human muscle by nuclear magnetic resonance (NMR) spectroscopy. The individual ^{31}phosphorus (^{31}P)-NMR spectra obtained on equal hemispherical tissue volumes (muscle plus skin and fat) were corrected for the thickness of the skin and of the subcutaneous fat. The volumes investigated were standardised using an external reference. The procedure described made possible the comparison of high energy phosphate concentrations among different subjects. It was applied to the assessment of [ATP] and [PC] in four groups of sedentary subjects (children, and adults aged 20–35, 35–50 and over 50 years), and in a group of athletes (volleyball players). The [ATP] and [PC] were not statistically different in the groups investigated.

Key words: Adenosine 5′-triphosphate – Phosphocreatine – Age – Training status – Method

Introduction

The determination of the absolute concentration of tissue high energy phosphates (adenosine 5′-triphosphate [ATP] and phosphocreatine [PC]), and of inorganic phosphate (P_i) is of paramount importance in the study of muscle energetics. Classical measurements rely on chemical assays (extraction, freezing and spectrophotometric analysis) of biopsy samples. These have been extensively employed for human studies also (e.g. Bergström 1967; Cheetham et al. 1986; Green et al. 1989; Harris et al. 1977; Jones et al. 1985; Karlsson and Saltin 1970; Karlsson et al. 1972; Larsson et al. 1978). The above techniques, however, only allow the assessment of the composition of very small samples and, in addition, cannot provide repeated measurements on the same muscle.

The ^{31}phosphorus nuclear magnetic resonance spectroscopy (^{31}P-NMRS) technique makes it possible to perform multiple determinations of high energy phosphates in vivo in the same muscle sample. Although ^{31}P-NMRS could theoretically provide absolute concentration (in millimoles per litre or millimoles per gram) values, so far it has proved impossible to perform such measurements in intact animals, because of the difficulty of detecting the precise size of the tissue volume investigated. As a consequence, the phosphate concentrations obtained by ^{31}P-NMRS have been mainly expressed either in relation to a known reference or as the ratio of two different molecules (mainly PC to P_i; e.g. Arnold et al. 1984; Chance et al. 1981, 1985, 1986; Laurent et al. 1991; McCully et al. 1988; Meyer et al. 1985; Taylor et al. 1983).

The aims of the present study were
1. To develop a ^{31}P-NMRS procedure for tissue volume normalisation in vivo that would allow comparison of high energy phosphate concentration values (in arbitrary units) among different subjects;
2. To determine in humans [ATP] and [PC] as a function of age and/or physical training.

Methods

Subjects. A group of 47 healthy male subjects aged 8–65 years participated in the study after giving their informed consent. Four age groups were selected among sedentary subjects. The fifth group consisted of 6 volley-ball players of international and national standard aged 24 (SD 3) years. The physical characteristics of the subjects are summarised in Table 1. The [ATP] and [PC] values that appear in the Results section (Table 2), are the mean of four observations (two spectra on each leg).

Anthropometric measurements of skinfold thickness. Skinfold thickness (skin plus subcutaneous fat) was measured by means of a skinfold caliper (Harpenden, Holtain Ltd, UK) on the posterior surface of both calves, above the triceps surae muscle at the site of contact of the coil with the limb surface. The skinfold thickness was then compared with the amount of fat detected from the ^{1}H-NMR spectra (see next paragraph).

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Table 1. Physical characteristics of the subjects

Group	Number of subjects	Age (year)		Height (cm)		Mass (kg)	
		mean	SD	mean	SD	mean	SD
A	10	28.8	3.4	174	3	73.0	6.6
B	9	40.7	4.2	179	6	80.6	5.6
C	9	57.7	4.9	174	6	74.0	9.7
D	13	10.9	2.3	142	10	35.8	7.7
E	6	25.8	3.2	190	8	82.2	8.2

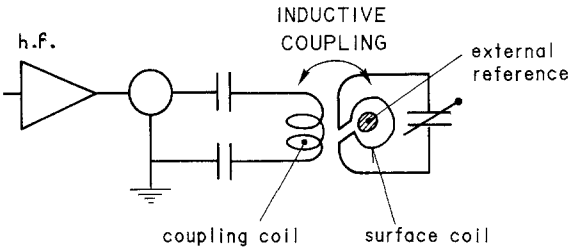

Fig. 1. Diagram of the coil arrangement within the magnet. *h.f.*, High frequency

Table 2. Values for adenosine 5′-triphosphate [ATP] and phosphocreatine [PC] concentrations (in arbitrary units) observed in the groups of subjects

		Children	Adults 20–35 years	Adults 35–50 years	Adults >50 years	Athletes
[ATP]	Mean	13.56	11.89	12.26	11.50	11.87
	SD	2.95	1.48	2.43	1.47	0.95
[PC]	Mean	47.13	44.66	46.73	45.18	45.61
	SD	10.42	5.97	7.82	5.63	3.51

^{31}P- and ^{1}H-NMR Spectroscopy. The measurements were made with a Brucker 2.35 T, 35-cm bore spectrometer. A double-tuned (^{1}H-^{31}P) 6-cm diameter radio-frequency coil (Decorps et al. 1985) was placed on the calf of the subject (at approximately one-third of the distance between the knee joint and the heel) in the homogeneous region of the magnetic field. The tissue mass investigated (about 70 cm^3) comprised, together with skin and subcutaneous fat, both gastrocnemius and soleus muscles in an approximately estimated ratio of 2 to 1. The use of an inductively coupled coil (Fig. 1) minimized di-electric losses of the signal due to tissue conductivity. The ^{1}H spectra for the assessment of water and fat were obtained from four free induction decays (FID) at a frequency of 100 MHz. Excitation time was 200 μs, relaxation delay 1 s. Such a short relaxation time allowed detection of water and fat peaks only.

The ^{31}P spectra were obtained on 40 FID. The shimming was performed on the proton signal. A pseudo 90° pulse angle, defined as the angle providing maximal signal intensity for ^{31}P, was chosen. Radio-frequency pulses lasted 40 μs whereas the relaxation delay was 15 s giving a total acquisition time for each spectrum of 10 min.

A reference phosphorus signal (a known amount of 200 $mmol \cdot l^{-1}$ methylene diphosphonate water solution in a glass capillary) was inserted at the centre of the coil. Such external reference was used to evaluate the homogeneity of the magnetic field and thus to correct the signals arising from the muscle for changes in the geometry of the system. This allowed calibration of the ^{31}P spectra, the peak areas of which were normalised to that of the reference tracing.

Since the investigated hemispherical tissue volume is constant, the muscle mass detected, and therefore the size of the ^{31}P-NMR signal will decrease with increasing thickness of the subcutaneous fat layer, i.e. the distance between the coil and the muscle mass. Therefore a separate set of experiments was carried out on a muscle phantom (2 kg of pork meat) whereby the relationship was established between the phosphorus signal, a function of the detected muscle volume, and the distance between the coil and the muscle mass. This distance was changed by inserting wooden plates of increasing thickness between the coil and the phantom. The acquisition of the spectra was performed as for the experiments on humans.

The assumption was made that free phosphorus metabolites in subcutaneous fat and in the skin are negligible and therefore do not contribute to the size of the signal.

Statistical analysis. Analyses of variance (ANOVA) were used for groups A, B, C to determine the effect of factor "age" on the parameters measured. For significant *F*-coefficients from ANOVA, posthoc analyses were carried out using the Fisher's protected least significant difference test to establish the differences between the means. Groups D and E were treated separately, each group being compared with group A using Student's *t*-test for unpaired observations. The accepted level of significance was set at 5%.

Results

An example of a ^{31}P-NMR spectrum with the external reference peak is shown in Fig. 2. The relationship between intensity of the phosphorus signals and the distance between the coil and the muscle phantom is shown in Fig. 3. The coil-to-muscle distance for humans (half of the skinfold thickness) was also found to correlate satisfactorily with homologous ^{1}H-NMR spectroscopy estimates of fat ($r=0.69$, $P<0.01$, Fig. 4).

The results as given in Fig. 3 allowed the normalization of [ATP] and [PC] to a given muscle volume. To achieve this, the observed [ATP] and [PC] values for any measured skinfold thickness were corrected to the peak area that would have been observed if skinfold thickness had been 0 mm.

The measured [ATP] and [PC] values normalised for muscle volume are summarised in Table 2. No significant difference could be found among groups.

Discussion

We are unaware of previous human ^{31}P-NMRS studies in which a direct comparison of [ATP] and [PC] obtained from different subjects has been performed. In fact, those studies in which the authors have not

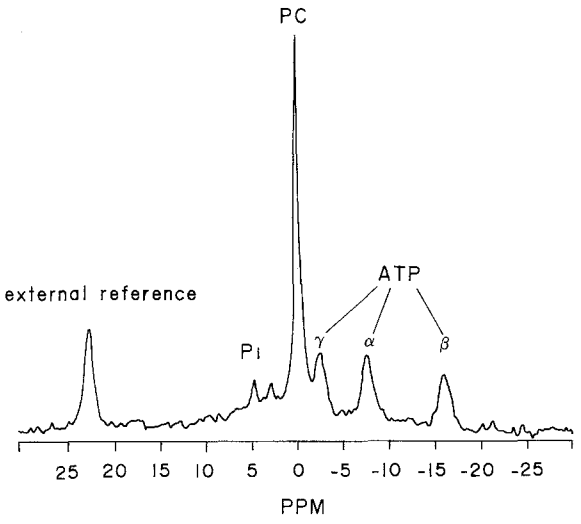

Fig. 2. Example ^{31}phosphorus-nuclear magnetic resonance (^{31}P-NMR) spectrum. *PC*, Phosphocreatine; *ATP*, adenosine 5′-triphosphate; *ppm*, parts per million

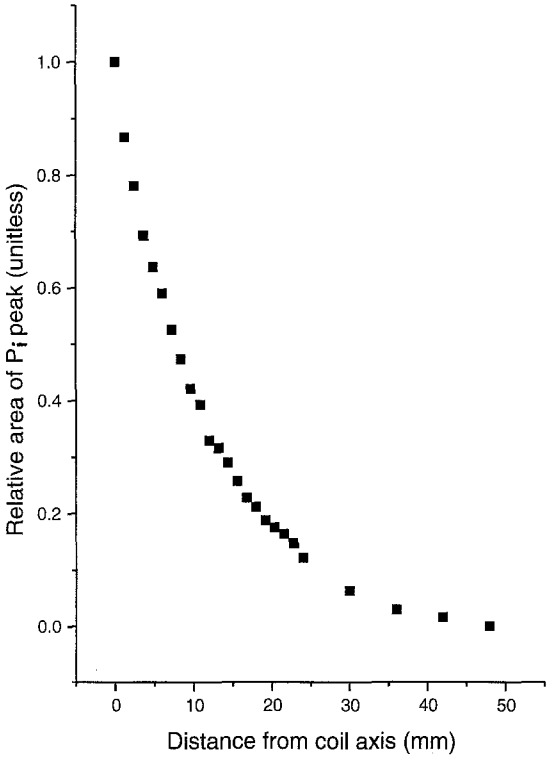

Fig. 3. Surface of inorganic phosphate (P_i) peak area as a function of the distance from the coil axis. The P_i values have been normalised with respect to the condition of no interface between coil and muscle (distance of coil axis of 0 mm) The *symbols represent experimental values* obtained on pig muscle after interposing wooden plates of increasing thickness between the coil and the muscle surface

looked at absolute high energy phosphates values (e.g. Meyer 1988, 1989; Meyer et al. 1985; Miller et al. 1988; Mole et al. 1985) were mainly concerned with the changes in [PC] within the same subject as a function of some independent variables, such as the imposed

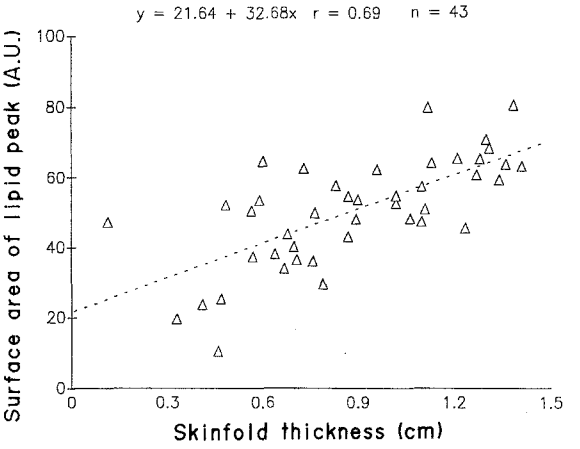

Fig. 4. Surface area of lipids on the ^{1}H-nuclear magnetic resonance spectrum as a function of skinfold thickness at the site of the measurements

mechanical load, or changes of the muscle metabolic status following physical or chemical changes. For such studies, a knowledge of muscle high energy phosphate concentration, even in arbitrary units, is not a prerequisite.

However, there are conditions, such as for example, the measurement of the kinetics of PC hydrolysis at the onset of constant load exercise in humans (see Binzoni et al. 1992) in which the knowledge of [ATP] and [PC], even in arbitrary units, is essential for the comparison of the rate of PC hydrolysis among different subjects. In such cases the important result is not the relative change of a given phosphorus metabolite as a function of time but only its absolute concentration difference. For the latter purpose a knowledge of the change, even in arbitrary (but identical) units, is required.

The [PC] and [ATP] values reported in this study are given in arbitrary units, and not in millimoles per litre, although an external phosphate reference was inserted at the centre of the coil. Despite the amount of phosphate inside the reference being known, a direct proportionality between the reference phosphate content and total muscle phosphates cannot be established, because the phosphorus nuclei in the former are in a physico-chemical configuration different from that in the latter. Indeed, the method would allow the assessment of the absolute concentrations of phosphates (in millimoles per litre) only if the external reference were uniformely distributed inside the muscle mass. It goes without saying that this condition cannot be fulfilled.

Despite its limitations, the present method provides for the first time a tool for estimating with sufficient precision the actual muscle volume detected by the coil (Fig. 3). This allows the standardization of the [ATP] and [PC] values obtained to a specific volume of muscle (approximately 70 ml in this study), from which a direct comparison among subjects can be made.

The present method was indeed based on an empirical calibration. The proposed method has been proved to work satisfactorily, as the internal variability of the

four measurements obtained in each of 47 subjects did not exceed 8%. This may still appear to be rather a rough estimate, but is better by far than that obtained using biochemical methods based on chemical assays of extremely small needle biopsy samples, especially if one considers that by the latter technique no timed measurements can be performed.

The results obtained by the present method showed that neither age nor training affected the concentration of high energy phosphates in human muscle. The finding of an equally high energy phosphate concentration in sedentary individuals and in athletic subjects confirms previous results from the literature that have been based on chemical assays (Eriksson 1980; Saltin and Gollnick 1983; Thorstensson et al. 1975). Having expanded the measurement of [ATP] and [PC] over a wide age range should prove extremely useful for future studies of peak muscle power of both normal individuals (see the accompanying paper, Ferretti et al. (1994)) and for future studies on patients with pathological muscle conditions.

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