Spatially resolved changes in diabetic rat skeletal muscle metabolism *in vivo* studied by ^{31}P -n.m.r. spectroscopy

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Phase-modulated rotating-frame imaging (p.m.r.f.i.), a localization technique for ^{31}P -n.m.r. spectroscopy, has been applied to obtain information on the heterogeneity of phosphorus-containing metabolites and pH in the skeletal muscle of control and streptozotocin-diabetic rats. Using this method, the metabolic changes in four spatially resolved longitudinal slices (where slice I is superficial and slice IV is deep muscle) through the ankle flexor muscles have been investigated at rest and during steady-state isometric twitch-contraction at 2 Hz. At rest, intracellular pH was lower, and phosphocreatine (PCr)/ATP was higher, throughout the muscle mass in diabetic compared with control animals. The change in PCr/ATP in diabetic muscle correlated with a decrease in the chemically determined ATP concentration. During the muscle stimulation period, the decrease in pH observed in diabetic muscle at rest was maintained, but not exacerbated, by the contractile stimulus. Stimulation of muscle contraction caused more marked changes in PCr/(PCr + P_i), PCr/ATP and P_i/ATP in the diabetic group. These changes were most evident in slice III, which contains the greatest proportion of fast glycolytic-oxidative (type IIa) fibres, in which statistically significant differences were observed for all metabolite ratios. The results presented suggest that some degree of heterogeneity occurs in diabetic skeletal muscle *in vivo* with respect to the extent of metabolic dysfunction caused by the diabetic insult and that regions of the muscle containing high proportions of type IIa fibres appear to be most severely affected.

INTRODUCTION

Chronic hypoinsulinaemia can be induced in rats by administration of the diabetogenic agent streptozotocin, leading to a disease state which shares many of the characteristics seen in untreated insulin-dependent diabetes mellitus (Rerup, 1970; Ganda *et al.*, 1976). A number of morphological, biochemical and biomechanical changes occur in skeletal muscle. In particular, changes in the activity or capacity of substrate transporter systems and changes in the maximal activities of key enzymes may lead to quantitatively significant changes in the major energy-metabolizing pathways of skeletal muscle (Wasserman & Vranic, 1986). In addition, the contractile properties of skeletal muscle may be affected, either as a consequence of changes in metabolic energy provision (Challiss *et al.*, 1989), or directly at the level of the contractile proteins and Ca²⁺ homeostasis (Armstrong *et al.*, 1975; Ganguly *et al.*, 1986).

Few studies have addressed the question of whether biochemical or biomechanical changes occur uniformly in all skeletal muscle fibre types of diabetic animals. However, where such investigations have been performed, some heterogeneity in enzyme activity changes (Chen & Ianuzzo, 1982; Noble & Ianuzzo, 1985), glycogen metabolism (Chen & Ianuzzo, 1982; Hamilton *et al.*, 1984) and contractile properties (Paulus & Grossie, 1983) have been observed; specifically, fast glycolytic-oxidative (IIa) fibres appear to be the most severely affected.

In a previous study, we applied a ^{31}P -n.m.r. localization technique, called phase-modulated rotating-frame imaging (p.m.r.f.i.), to investigate the spatial heterogeneity of high-energy phosphate metabolism in the ankle flexor muscles of the rat (Challiss *et al.*, 1988). Here we extend the application of this technique to the investigation of skeletal muscle metabolism in streptozotocin-diabetic rats *in vivo*, both at rest and during steady-state contraction of the ankle flexor muscles.

METHODS

Animal preparation

Male Wistar rats weighing 130–160 g (Harlan-OLAC, Bicester, Oxon, U.K.) were maintained in the Departmental animal facility for at least 5 days before induction of diabetes.

Procedures have been described previously (Challiss *et al.*, 1989). Animals were anaesthetized, and either 1 ml of a freshly made solution of streptozotocin (60 mg/ml)/50 mM-citrate buffer (pH 4.5)/kg, or an equivalent volume of citrate buffer vehicle, was injected into the right femoral vein. After suturing the wound, animals were allowed to recover and were given free access to food and water. All animals given streptozotocin developed a marked hyperglycaemia and glucosuria within 48 h of injection.

^{31}P -n.m.r. studies were performed 20–22 days after induction of diabetes or injection of vehicle. At this time, control animals weighed 187 ± 7 g and diabetic animals weighed 164 ± 6 g. Animal preparation for ^{31}P -n.m.r. spectroscopy and unilateral muscle stimulation of the left leg was performed as described previously (Challiss *et al.*, 1986, 1988). The distal tendons of the gastrocnemius-plantaris-soleus muscles were attached to a force transducer and the muscle was adjusted to optimal length for maximal isometric contraction (Shoubridge *et al.*, 1984). The radio-frequency transmitter and receiver coils were placed in position (Challiss *et al.*, 1988) and the Perspex cradle containing the animal was placed into the vertical bore of the magnet. Throughout the experiment, core body temperature was maintained at 37 ± 1 °C and anaesthesia was maintained by delivery of 0.5–1 % halothane in N₂O/O₂ (1:1) via a face mask.

^{31}P -n.m.r. spectroscopy

All experiments were performed using a 4.3 T vertical bore magnet, interfaced to a Bruker Biospec spectrometer. The

Abbreviations used: p.m.r.f.i., phase-modulated rotating-frame imaging; PCr, phosphocreatine.

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p.m.r.f.i. technique has been described in detail previously (Blackledge *et al.*, 1987; Challiss *et al.*, 1988). The orthogonal double-coil system ensures that the B_1 field gradient is linear in the reception region and that the B_1 isocontours are flat. The two coils are completely isolated during transmission and receiving (Blackledge *et al.*, 1987), and the size of the coils ensures homogeneous collection of signal from a region defined as 0.2–0.8 transmitter coil radii from the transmitter coil. Although the resolution of each experiment is influenced by a number of variables (e.g. transmitter tuning, optimization of pulse width), a resolution of about 1 mm was generally achieved (Blackledge *et al.*, 1986).

Each image was acquired from 24 incremental pulse widths, each of which represented 64 transients, using a relaxation delay of 3 s. Consequently, the total accumulation time was 75 min. After acquisition of a resting muscle image, muscle contraction at a frequency of 2 Hz was caused by sciatic nerve stimulation (supramaximal stimulation to cause 100% muscle fibre recruitment). After 20 min, muscle twitch tension was constant and phosphocreatine (PCr), ATP, P_i and intracellular pH were in a steady state. At this time, a second image was collected.

Experimental details of the two-dimensional data processing have been described elsewhere (Blackledge *et al.*, 1987). Line broadening (10 Hz) was applied in the f_0 dimension. The pulse increment was nominally set at 90° at the centre of the sample, as was the phase-encoding pulse. Data were zero-filled to give 32 positive (f_1) slices; however, the number of slices containing ^{31}P information varied in the range 10–20. For this reason, each data set was divided into four slices of equal width (I–IV), each obtained by co-addition of data from 2–4 slices from the original matrix and, therefore, representing approx. 3–4 mm longitudinal slices through the muscle mass. For both experimental groups, slice I represents predominantly superficial muscle overlying the gastrocnemius, and slice IV represents the deep ankle flexor muscle, with a small contribution from the ankle-extensor muscle lying close to the tibia.

Localized longitudinal relaxation time (T_1) measurements were made using the p.m.r.f.i.–i.r. technique (Blackledge & Styles, 1989). The pulse sequence used for determination of T_1 values was:

$$2\lambda_{\pm x} - \tau - n\Delta\theta_{\pm x} - \lambda_y - \text{acquisition} - \text{relaxation delay},$$

where λ and $\Delta\theta$ are both equal to $\pi/2$ at the sample centre and vary between $3\pi/4$ to $\pi/4$ from the front to the back of the sample (Blackledge & Styles, 1988, 1989). Eight τ times were employed from 0.1 to 20 s, with a recycle delay of 20 s. An image comprising 2×2 scans per increment was collected for each inversion

recovery delay time τ . Two data sets were collected for each image, $\Delta\theta_{\pm x}$ and combined during the 2-dimensional Fourier transform processing to remove the phase-twist incurred in the matrix. Ten increments were collected for each image, the spatial resolution being calibrated for this protocol as 3 mm. Although steady-state muscle contraction at 2 Hz was occurring throughout the acquisition period (80 min), each τ -image was interleaved, so that any temporal instability would be averaged over all τ values. In addition, for each experiment it was shown that the metabolic steady state was well maintained by comparison of spectra acquired before and after T_1 data acquisition.

Resonance intensities were determined by cutting the P_i , PCr and ATP γ -P peaks from plotted spectra and weighing them. These values were confirmed by using the integration routine in the Bruker Biospec I software. Intracellular pH was calculated from the chemical shift difference between the P_i and PCr resonances (Gadian *et al.*, 1979). As previously discussed, ratios are expressed relative to the ATP γ -P due to off-resonance effects distorting the spatial position of the ATP β -P (Blackledge *et al.*, 1986).

Analysis of other metabolites

In a separate series of experiments, four groups of animals were anaesthetized and given intravenous injections of 50, 60 or 75 mg of streptozotocin/kg body wt. or citrate buffer vehicle as described above. After 21 days, animals were anaesthetized (60 mg of sodium pentobarbital/kg body wt., intraperitoneal) and blood samples were taken for glucose (Bergmeyer *et al.*, 1974) and fatty acid (Okabe *et al.*, 1980) determination. The ankle flexor muscles were exposed and the gastrocnemius muscle was freeze-clamped. Adenine nucleotides, creatine and PCr concentrations were determined by h.p.l.c. analysis of muscle extracts (Harmsen *et al.*, 1982).

RESULTS AND DISCUSSION

The feasibility of acquiring spatially resolved ^{31}P -n.m.r. spectra from the ankle flexor muscles of the rat using the p.m.r.f.i. technique has been demonstrated previously (Blackledge *et al.*, 1986; Challiss *et al.*, 1988). The ankle flexor muscle group provides a good experimental model, as there is a gradation of fibre types across the muscle mass; thus, superficial muscle contains more than 75% type IIb fibres, whereas deep muscle contains more than 50% type IIa fibres with a significant proportion of type I fibres (Armstrong & Phelps, 1984).

Administration of streptozotocin (60 mg/kg body wt.) caused

Table 1. Effects of administration to rats of increasing doses of streptozotocin on plasma glucose and fatty acid, and on gastrocnemius muscle ATP and total creatine concentrations

Concentrations of ATP and total creatine were obtained by h.p.l.c. analysis (Harmsen *et al.*, 1982). Values are presented as means $\pm$ S.E.M. for four to six animals in each group. Statistically significant differences (Student's *t* test) are indicated as ** $P < 0.01$, *** $P < 0.001$ for diabetic versus control groups, and † $P < 0.05$ for diabetic group 1 versus diabetic group 3.

	Control	Diabetic groups		
		1	2	3
Streptozotocin dose (mg/kg)	0	50	60	75
Concentration in plasma (mM):				
Glucose	5.1 $\pm$ 0.4	24.7 $\pm$ 1.5***	23.7 $\pm$ 2.4***	26.8 $\pm$ 3.7***
Fatty acid	0.23 $\pm$ 0.06	0.71 $\pm$ 0.09**	1.23 $\pm$ 0.21***	1.27 $\pm$ 0.19***
Concentration in gastrocnemius muscle ($\mu\text{mol/g}$ of tissue):				
ATP	7.11 $\pm$ 0.24	7.23 $\pm$ 0.16	6.64 $\pm$ 0.29	6.43 $\pm$ 0.22†
Total creatine	27.1 $\pm$ 0.6	26.1 $\pm$ 1.7	26.9 $\pm$ 1.3	26.1 $\pm$ 1.5

Fig. 1. ^{31}P -n.m.r. stack plots of data from resting (a) and exercising (b) leg muscle of a streptozotocin-diabetic rat

Chemical shift is shown along the x-axis and distance from the transmitter radiofrequency coil is shown along the y-axis. Total acquisition time for each image was 75 min and both images were acquired from the same animal. Resonances are PCr (a), ATP γ -P (b), ATP α -P (c) and P_i (d). Image (b) was collected during steady-state stimulation (commencing 20 min after initiation of muscle contraction) at 2 Hz.

overt symptoms of uncontrolled diabetes within 48 h in all animals. At the time of study (21 days), blood glucose concentrations were about 4-fold above control levels and plasma fatty acid concentrations were significantly elevated (Table 1).

Spectral images of phosphorus metabolites *in vivo* in resting and stimulated distal leg muscles of a diabetic rat are shown in Fig. 1. We have previously shown that resting metabolite levels in the ankle-flexor muscles of diabetic rats are not significantly

Fig. 2. Intracellular pH for four spatially resolved longitudinal slices through ankle flexor muscles of control and diabetic rats

Intracellular pH was determined from the chemical shift of P_i relative to PCr (Gadian *et al.*, 1979) for spectra obtained using the p.m.r.f.i. technique, where slice I is superficial muscle and slice IV is deep muscle (see the Methods section). Values were obtained from spectra acquired from control (□) or diabetic (■) rats at rest (a) or during muscle contraction at 2 Hz (b) and are shown as means $\pm$ S.D. for five or six experiments in each group. Statistically significant differences between control and diabetic groups (Student's *t* test) for each spatially resolved region are shown as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

different from those observed *in vivo* in age-matched or weight-matched euinsulinaemic rats. However, some evidence for pH differences at rest has been reported (Challiss *et al.*, 1989), which was suggestive of pH heterogeneity in diabetic skeletal muscle. Data for spatially resolved intracellular pH in ankle flexor muscles of control and diabetic rats are shown in Fig. 2. At rest, control animals show a small pH gradation across the muscle mass, with superficial muscle being relatively more acidic than deep muscle (region I, 7.02 ± 0.01 ; region IV, 7.09 ± 0.08). For all regions, diabetic muscle exhibited lower resting pH values, with the differences reaching statistical significance for regions II and III (Fig. 2a). However, the pH differences observed for diabetic rats between superficial and deep longitudinal slices are no more pronounced than in control animals.

The tendency for the $\text{PCr}/(\text{PCr} + \text{P}_i)$ ratio to decrease and P_i/ATP to increase in deeper regions, previously reported for control animals (Challiss *et al.*, 1988), is confirmed in the present study, and a similar gradation of changes is observed for the diabetic group (Figs. 3a and 3c). In contrast with previous findings (Challiss *et al.*, 1988), PCr/ATP ratios were greater in diabetic than in control animals throughout the interrogated muscle group (Fig. 3b). Although no effect of streptozotocin-diabetes on either ATP or PCr concentration was reported in the previous study (Challiss *et al.*, 1989), data given in Table 1 demonstrate a dose-dependent effect of streptozotocin on gastrocnemius muscle ATP concentration. A small decrease in ATP concentration was observed when the dose of streptozotocin was increased to 60 mg/kg body wt., with the effect reaching statistical significance at the higher dose (Table 1). No effect of the increasing severity of diabetes was observed on the total creatine concentration. Therefore the decrease in ATP concentration observed may explain the increase in PCr/ATP (Fig. 3b).

Biomechanical performance was monitored during muscle stimulation. For the control group, initial twitch-tension was 1.67 ± 0.23 g/g body wt., and this value declined to a steady-state value of 1.40 ± 0.17 g/g body wt. during the data acquisition period at 2 Hz muscle stimulation. For the diabetic group, initial twitch tension was 1.31 ± 0.24 g/g body wt.; this apparent decrease compared with the control group could be accounted for by the lower body weight of the diabetic group and the decrease in the relative ankle flexor muscle/body wt. ratio (Challiss *et al.*, 1989). For the diabetic group, twitch-tension was maintained at 80% of the initial tension development for the data acquisition period at 2 Hz muscle stimulation.

The slight gradation of pH across the interrogated muscle mass observed for both control and diabetic animals at rest was maintained during muscle stimulation (Fig. 2b). Although the pH of diabetic muscle was significantly lower than that of control muscle in all regions, the relative change from rest to steady-state twitch contraction was similar in both groups, supporting the proposal that the decreased pH observed at rest is not exacerbated by the increased metabolic demand of contraction at modest stimulation frequency (Challiss *et al.*, 1989).

Sciatic nerve stimulation at a frequency of 2 Hz caused marked changes in PCr and P_i concentrations, with new constant levels of these metabolites being achieved within 10–15 min of initiation of muscle contraction (Challiss *et al.*, 1987). For control animals, the decrease in $\text{PCr}/(\text{PCr} + \text{P}_i)$ was greatest in superficial muscle, with smaller changes being observed in deeper muscle (Fig. 3a; Challiss *et al.*, 1988). Similar changes in these metabolites were observed in superficial muscle of diabetic animals; however, the change in $\text{PCr}/(\text{PCr} + \text{P}_i)$ was more uniform in the superficial and deep muscle of the diabetic group, and consequently significantly greater changes between control and diabetic groups were observed in deep muscle during contraction (Fig. 3a). Comparable differences were observed

Fig. 3. $\text{PCr}/(\text{PCr} + \text{P}_i)$ (a), PCr/ATP (b) and P_i/ATP (c) for four spatially resolved longitudinal slices through ankle flexor muscles of control and diabetic rats

Metabolite ratios were determined from spectra obtained using the p.m.r.f.i. technique (see the Methods section). Data are shown as means $\pm$ s.d. for five or six experiments for control ($\circ$, $\square$) and diabetic ($\bullet$, $\blacksquare$) rats studied at rest ($\circ$, $\square$) or during muscle contraction at 2 Hz ($\bullet$, $\blacksquare$). Statistically significant differences between control and diabetic groups (Student's *t* test) during muscle contraction for each spatially resolved region are shown as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table 2. T_1 values for PCr and P_i in ankle flexor muscle of control and diabetic rats

Values are presented as means $\pm$ S.E.M. for three determinations in each group.

	T_1 (s)	
	PCr	P_i
Control	3.93 $\pm$ 0.18	6.32 $\pm$ 0.12
Diabetic	4.33 $\pm$ 0.14	6.30 $\pm$ 0.18

between the two experimental groups with respect to P_i /ATP (Fig. 3c). Differences in the PCr/ATP ratio observed between the two experimental groups during muscle contraction were less clear because of the higher PCr/ATP values seen in the diabetic group at rest (see above). For the control group, the decrease in PCr/ATP during contraction was $36.6 \pm 1.1\%$, whereas the comparable decrease for the diabetic group was $48.6 \pm 1.4\%$ ($P < 0.01$). The greatest difference was observed for slice III, in which a significant decrease was observed in the diabetic animals which was independent of the resting difference in PCr/ATP between the two groups (Fig. 3b).

Therefore these data are in agreement with previous findings which have suggested that skeletal muscle containing a greater proportion of type IIa fibres are more severely affected by the diabetic state (Armstrong *et al.*, 1975; Chen & Ianuzzo, 1982; Paulus & Grossie, 1983; Hamilton *et al.*, 1984).

A possible criticism of the data obtained using the p.m.r.f.i. method is that spectra are collected with a pulse-delay of 3 s, and therefore full relaxation does not occur between excitation-acquisition cycles (Challiss *et al.*, 1988). We have therefore attempted to determine whether the changes in metabolite ratios detected in the diabetic muscle can be accounted for by differences in longitudinal relaxation times (T_1) between the two experimental groups. Localized T_1 measurements were made using the p.m.r.f.i.-i.r. technique (Blackledge & Styles, 1989). This novel method allows separate measurements of T_1 to be performed simultaneously from localized volumes throughout the receiver region with optimal sensitivity. The method has enhanced accuracy compared with the standard inversion-recovery technique (Vold *et al.*, 1968), as the localized data are differentiated with respect to the applied pulse angle due to the B_1 -dependence of the p.m.r.f.i. experiment. The uncertainty, and additional induced error, associated with the inversion and acquisition pulses in the standard inversion recovery method are therefore removed (see Blackledge & Styles, 1989, for further details).

Data were collected from slices I-IV defined in the p.m.r.f.i. protocol. No significant differences in relaxation times were noted with respect to spatial position, and therefore the T_1 values presented in Table 2 are means of the averaged data for each animal studied. No significant differences in T_1 values for PCr or P_i were observed between control and diabetic animals. Therefore differences observed between the two experimental groups with respect to metabolic ratios are a true reflection of altered metabolism and are not a consequence of the n.m.r. method used.

In all experiments, spectra were collected 30 min after termination of muscle contraction. For control animals, spectra

collected after either p.m.r.f.i. or p.m.r.f.i.-i.r. protocols were quantitatively comparable with spectra collected at the start of the experiment. For diabetic animals, PCr and P_i concentrations returned to pre-stimulation values; however, as reported previously (Challiss *et al.*, 1989), a small decrease ($17 \pm 7\%$) in ATP concentration was caused by the stimulation protocol.

In summary, a chronic diabetic state caused by administration of 60 mg of streptozotocin/kg body wt. resulted in a significant decrease in muscle intracellular pH and a small decrease in ATP concentration which appeared to be essentially independent of muscle fibre composition. During muscle contraction, the difference in muscle pH between experimental groups was maintained; however, spatial resolution of changes in PCr/(PCr+ P_i), P_i /ATP and PCr/ATP demonstrated that greater differences between control and diabetic muscle were observed in deeper muscle, which contains a greater proportion of type IIa fibres.

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