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A non-invasive selective assessment of type I fibre mitochondrial function using ^{31}P NMR spectroscopy

Evidence for impaired oxidative phosphorylation rate in skeletal muscle in patients with chronic heart failure

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Background Skeletal muscle abnormalities contribute considerably to the clinical expression of heart failure. Deconditioning, underperfusion and an increased number of type IIb glycolytical fibres lead to early lactate production and muscle fatigue at low exercise levels. Aerobic muscle metabolism may also be impaired, as suggested by biopsy studies. Thus far, no data are available from non-invasive studies to indicate the extent of aerobic muscle dysfunction during low-grade exercise which does not induce acidosis.

Methods and results Mitochondrial function of skeletal muscle during fibre type I activation was studied in 22 patients with chronic heart failure [NYHA class III, left ventricular ejection fraction $28 \pm 2\%$, (patients)] on ACE inhibitors, diuretics and digoxin, and in 20 normal subjects, using ^{31}P NMR spectroscopy of a single right forearm flexor muscle during three mild intermittent exercise levels (0–40% of maximum voluntary contraction) and recovery time. At rest, the inorganic phosphate/phosphocreatine ratio was different [$0{\cdot}13 \pm 0{\cdot}005$ (patients) vs $0{\cdot}09 \pm 0{\cdot}002$ (normal subjects), $P = 0{\cdot}0001$]. However, intracellular pH was comparable. Local acidosis (tissue pH <6·9) was avoided to prevent fibre type IIb activation. Calculated resting phosphate potential levels were comparable, but the slope and intercept of the linear relationship of phosphate potential and workload were significantly lower in patients than in normal subjects ($11{\cdot}7 \pm 0{\cdot}7$ vs $15{\cdot}8 \pm 0{\cdot}6$ and 139 ± 7 vs 196 ± 7, patients vs normal subjects, indicating early exhaustion of intracellular energy at lower exercise levels. Also, maximum calculated workload at which tissue ADP stabilized was lower in patients than in normal subjects ($88 \pm 7\%$ vs $120 \pm 4\%$ of maximum voluntary workload, patients vs normal subjects, $P < 0{\cdot}05$). Time to recovery to pre-test phosphocreatine levels was prolonged by 46% in patients compared to normal subjects ($P < 0{\cdot}05$).

Conclusions In heart failure, oxidative fibre mitochondrial function in skeletal muscle is impaired, as reflected by the reduced phosphate potential and oxidative phosphorylation rate, early exhaustion and slowed recovery of intracellular energy reserve at workloads, which do not affect intracellular pH.
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Key Words: Heart failure, mitochondrial function, ^{31}P NMR spectroscopy, skeletal muscle, phosphorylation rate, muscle fibre type.

Introduction

Reduced exercise tolerance is a typical clinical feature of the heart failure syndrome. Several studies have suggested that besides deconditioning and underperfusion, secondary to the reduction in cardiac pump function, intrinsic changes in peripheral skeletal muscle contribute to this phenomenon[1]. Changes in the fibre type composition of skeletal muscle, and abnormalities in the energy metabolism in skeletal muscle cells have been described in 'in vitro' studies[2]. In vivo investigations in muscle biopsy specimens have also suggested that oxidative ATP synthesis is impaired in heart failure patients. Moreover, mitochondrial crista volumes are reduced, which suggests mitochondrial dysfunction[3].

^{31}P NMR studies during exercise in patients with heart failure have demonstrated abnormal metabolic changes, such as early acidification, increased

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phosphocreatine utilization and concomitantly increased inorganic phosphate levels. Moreover, recovery to pre-exercise phosphocreatine levels is slowed in some patients[4–7]. As yet, the extent to which this mitochondrial dysfunction contributes to the symptomatology of heart failure is unclear. To address this issue 'in vivo', using ^{31}P NMR spectroscopy techniques, it is required that the oxidative capacity of a homogeneous population of fibre types and, consequently, of comparable mitochondrial content is studied. Results from ^{31}P NMR studies investigating mitochondrial function reported so far have been ambiguous as they did not meet this requirement since both oxidative and glycolytic fibres were recruited in the voluntary exercise regimens used[6]. In studies conducted on forearm flexor muscles and employing voluntary handgrip exercise, this could be attributed to poor control over the mechanical workload on individual fibres within the population of fibres under investigation[8–11]. Recently, an experimental design for ^{31}P NMR measurement of the work–cost relationship in single human finger flexor muscle was described in which the mechanical workload could be carefully controlled, thus allowing selective recruitment of only oxidative fibres within the sampled muscle mass[11].

In the present study, we applied this experimental design to measure the variation in phosphate metabolite concentrations and pH at different levels of muscle power output in oxidative fibres of forearm muscle of patients with stable, moderate heart failure. Our results indicate that mitochondrial dysfunction does contribute to the reduced exercise tolerance of skeletal muscle in heart failure.

Methods

Patients

After informed consent and after approval of the protocol by the local Ethical Review Committee, 20 male and two female patients with heart failure NYHA class III and a left ventricular ejection fraction $\leq$45% participated in the study. All patients had been on stable ACE inhibitor and diuretic therapy for at least 4 weeks before nitrates were withdrawn the day before the study; other vaso-active medication was stopped 3–5 days pre-study, depending on plasma half-lives. Control data of forearm skeletal muscle bioenergetics were obtained from 20 healthy untrained volunteers. Fifteen subjects were male, five female, mean age 22 years (range 8–40 years). The ^{31}P NMR data of this group are reported elsewhere[12]. Patient characteristics are shown in Table 1.

Table 1 Patient characteristics

Number of patients	22
Sex	20 males, 2 females
Age	63 ± 2 years (range 43–77 years)
NYHA class	III
Previous infarction	15
Idiopathic cardiomyopathy	7
LV ejection fraction	26 ± 2% (range 10–44%)

Controls: 20 healthy, untrained volunteers, aged 8–40 years.

NMR measurements

^{1}H MR images and ^{31}P NMR spectra were obtained using a Philips S15 HP whole body NMR spectrometer (Eindhoven, The Netherlands) operating at 1·5 T (clear bore diameter after removal of the body coil: 70 cm). Positioning of the patients and localization of the flexor digitorum profundus muscle of the right forearm was performed using magnetic resonance imaging. ^{31}P NMR spectra were obtained by collecting data from 50 exercise cycles at each workload, using a 400 ms window during exercise. Data from the spectrum before and after the contracted state of the muscle were discarded. Data processing was performed off-line.

Exercise protocol

The exercise protocol and equipment have been described elsewhere[10]. Briefly, exercise involved bulb-squeezing with visual feedback of power-output on an audio-signal using only the tips of the fourth and fifth digit at three steady-state workloads normalized to maximum voluntary contraction in a ramp protocol. Rather than using fixed workloads for all, the load of the second and third work level for each individual were set by the supervising physician by monitoring on-line the metabolic response to the first, lowest workload to meet each subject's individual oxidative capacity. The frequency of bulb-squeezing was four times per minute. Power output was measured and recorded for calculation of actual power output at each workload. At the end of each protocol, τ, the time to recovery to pre-study phosphocreatine levels, was measured.

Calculation of metabolite concentrations

The ATP and total creatine concentrations were assumed to be identical for normal subjects and patients. The mean values of these metabolites reported for human upper leg muscle (8·2 mM and 42·7 mM, respectively)[13] were used in our calculations. The phosphate (Pi) and phosphocreatine (PCr) concentrations in the resting state were calculated from the measured ratios over ATP, using saturation correction factors of 1·2 and 1·4, respectively, determined from fully relaxed spectra. The average free ADP concentration in fibres within the sampled muscle mass was calculated from the creatine kinase equilibrium according to the equation:

$$[ADP]=[ATP][Cr]/(1{\cdot}66\times10^{9})(10^{-pH})[PCr] \quad (1)$$

where $1{\cdot}66 \times 10^9$ is the equilibrium constant for creatine kinase reported by Veech[14] and [Cr] is the creatine concentration. From these values, the concentration-dependent term ln([ATP]/[ADP][Pi]) in M^{-1} of the free energy of ATP hydrolysis (ΔG_p) or phosphate potential was calculated.

Determination of oxidative phosphorylation capacity

Two measures of the muscle's capacity for oxidative phosphorylation were obtained based on the steady-state equations relating to cellular ATP consumption rate (dominated by ATPase rate) and ATP synthesis rate (dominated by mitochondrial ATPase rate at constant pH 7·0). The derivation of the steady-state equations that were used has been described previously[12]. The first equation concerns a linear function of the phosphate potential vs steady-state power output data of the form:

$$P = \mathbf{m} \times \ln([ATP]/[ADP][Pi]) + \mathbf{b} \quad (2)$$

where **m** and **b** are parameters containing kinetic proportionality and proton-coupling stoichiometry constants of oxidative phosphorylation (dimensions: % maximum voluntary contraction × $mol.l^{-1}$ and % maximum voluntary contraction, respectively). The absolute value of **m** proportionally reflects the cellular oxidative phosphorylation capacity and was used as the first 'in vivo' measure of the latter[15]. Secondly, an estimate of the maximal steady-state power output (which is oxidative ATP fuelled) was obtained for each subject from the fit of a hyperbolic function to the [ADP] vs steady-state power output data of the form:

$$P = C1 \times [ADP]/[ADP] + Km) - C2 \quad (3)$$

where P is the steady-state power generated by muscle fibres (maximum voluntary $contraction^{-1}$), C1 and C2 are parameters reflecting maximal and basal oxidative phosphorylation rate (dimension: both in % maximum voluntary contraction) and Km is the [ADP] at half-maximal respiration rate (in μM) for the given boundary condition of the total adenine nucleotide and creatine content of the flexor digitorum profundus muscle. At high [ADP] values, the function given by the equation approaches a value of (C1–C2), the maximal steady-state power output (% maximum voluntary contraction) which was used as the second measure of the cellular capacity for oxidative phosphorylation.

Statistical calculations

ANOVA linear and non-linear curve fitting were performed using SAS/STAT® software (version 6·10). All data are presented as mean ± standard error of the mean. Unpaired Student's t-tests were performed on measurement comparisons between groups where a *P*-value $\leq 0{\cdot}05$ was considered statistically significant.

Results

Accurate, MRI-guided, surface coil placement on the superficial region of the flexor digitorum profundus, corresponding to fibres involved in flexing of the fourth and fifth digit, was achieved in all patients. Also, exercise was completed by all patients according to protocol. In the resting state, phosphocreatine levels were lower in patients than in normal individuals ($33{\cdot}4 \pm 0{\cdot}5$ vs $36{\cdot}6 \pm 0{\cdot}6$ mM, respectively) and phosphate levels were higher ($4\,3 \pm 0{\cdot}2$ vs $3{\cdot}2 \pm 0{\cdot}1$ mM, respectively). Consequently, the phosphate/phosphocreatine ratio was different ($0{\cdot}13 \pm 0{\cdot}005$ in patients vs $0{\cdot}09 \pm 0{\cdot}002$ in normals). Intracellular pH in patients was similar to that in normal individuals ($7{\cdot}05 \pm 0{\cdot}03$ vs $7{\cdot}06 \pm 0{\cdot}02$, respectively). During exercise, the magnitude of changes in phosphate and phosphocreatine was greater in patients, as illustrated in Fig. 1, which shows a typical series of ^{31}P NMR spectra obtained from forearm muscle of a control and a patient at identical power output levels. Importantly, intracellular pH decreased only to $6{\cdot}96 \pm 0{\cdot}11$ at the highest workload both in normal individuals and in patients. This is also illustrated in Fig. 1, by the observation that the line widths of phosphate and phosphocreatine were similar at all power output levels. In seven patients, intracellular pH intermittently dropped to low values (6·95–6·71) during the first workload, but recovered to the normal range of values at subsequent workloads. Data from these workloads were excluded from the analysis. Figure 2 shows the linear correlation of the concentration-dependent term of the phosphate potential (ln(ATP/ADP × Pi), and power output predicted by equation (2) for the pooled data of all 22 patients ($r^2 = 0{\cdot}72$). At zero workload, i.e. the resting state, the phosphate potential was comparable in both groups. Individual line fitting used to calculate slope **m**, had corrected r-square values ranging from 0·86 to 0·99. Compared to normal individuals, both the average slope **m** and intercept **b** were significantly lower, $11{\cdot}7 \pm 3{\cdot}0$ % maximum voluntary contraction × M and 139 ± 8 % maximum voluntary contraction in patients than in normal individuals ($15{\cdot}8 \pm 2{\cdot}5$ % maximum voluntary contraction × $mol.l^{-1}$ and 196 ± 7 % maximum voluntary contraction). Average values for **m** and **b** in patients were approximately 70% of normal phosphorylation rates. Figure 3 shows the variation of the average intracellular [ADP] in fibres with power output for the pooled data of all 22 patients. In patients, higher intracellular [ADP] levels are associated with lower power output levels compared to normal individuals. In addition to the phosphorylation rates, the average C1 value for maximal O_2 consumption, estimated for each individual separately, differed significantly, 57 ± 20 % maximum voluntary contraction in

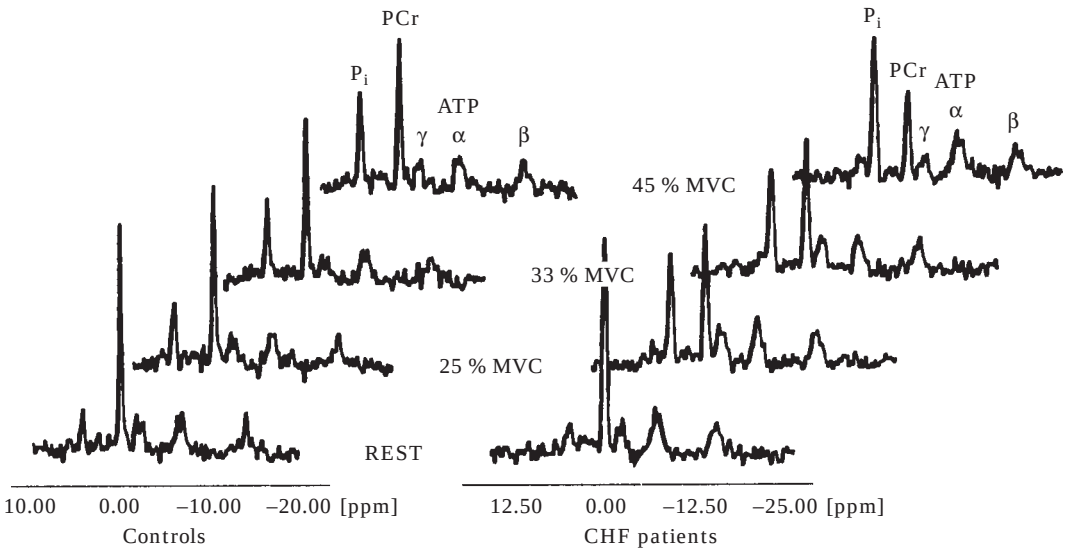

Figure 1 **Presentation of ^{31}P NMR spectra taken at rest and during exercise from the flexor digitorum profundus muscle in patients with chronic heart failure and normal untrained individuals. Note the rapid decline in the phosphocreatine (PCr) peak and the rapid incline in the inorganic phosphate peak (Pi) in heart failure patients compared to normals, already at very low exercise levels. Also, at the highest exercise level, the peak widths are still narrow, which is indicative of stable intracellular pH.**

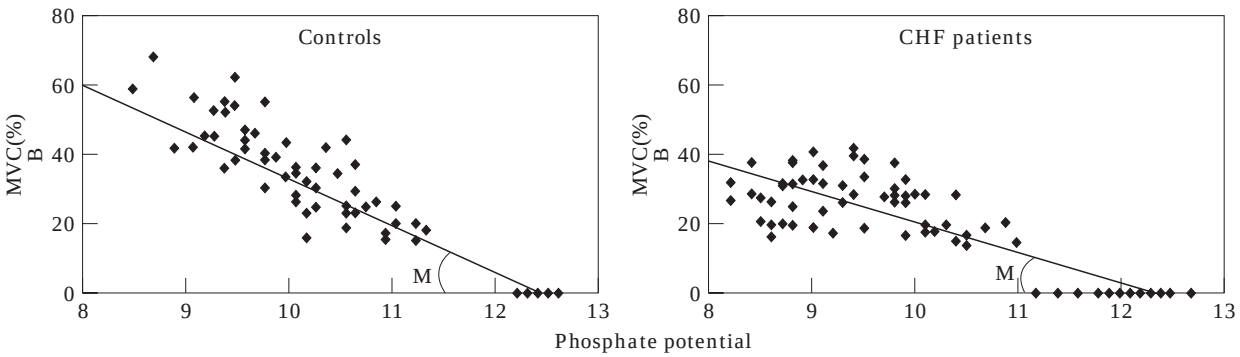

Figure 2 **Graph showing the linear relationship between the phosphate potential and maximum voluntary contraction (MVC). Note that the slope m and intercept b are significantly lower in heart failure patients (CHF) compared to normal individuals (controls), which is indicative of lower phosphorylation rates. At zero workload the average values for the phosphate potential are comparable in both groups.**

patients vs 120 ± 17 % maximum voluntary contraction in normal individuals, a decrease of 53%. However, values at zero workload were comparable in both groups. The C2 value, indicative of basal O_2 consumption, was comparable in both groups (29 ± 1 in normals vs 25 ± 4 % maximum voluntary contraction in patients). In contrast, maximal steady-state power output (C1–C2), calculated for each individual from the fit of the equation (3) to the [ADP] vs power output data, was lower in patients (63 ± 3 vs 91 ± 3 % maximum voluntary contraction in normals). As Fig. 4 shows, there is a positive linear correlation between slope **m** and (C1–C2). Adjusted r^2 for this fitted line is 0·74. Along this line, a group-wise distribution of normal subjects and heart failure patients can be noticed, where heart failure patients are associated with the domain corresponding to lower mitochondrial function. Individual data are displayed in Table 2. The calculated time constant τ of phosphocreatine resynthesis after exercise was found to be significantly prolonged in the group of heart failure patients, 65 ± 19 s vs 42 ± 11 s in normal subjects, a prolongation of 55%.

Discussion

In the present study of skeletal muscle bioenergetics in heart failure we observed a significantly altered work–energy cost relationship of forearm finger flexor muscle of patients with stable, moderate heart failure. Importantly, the magnitude of changes in phosphocreatine and phosphate levels and in intracellular pH to

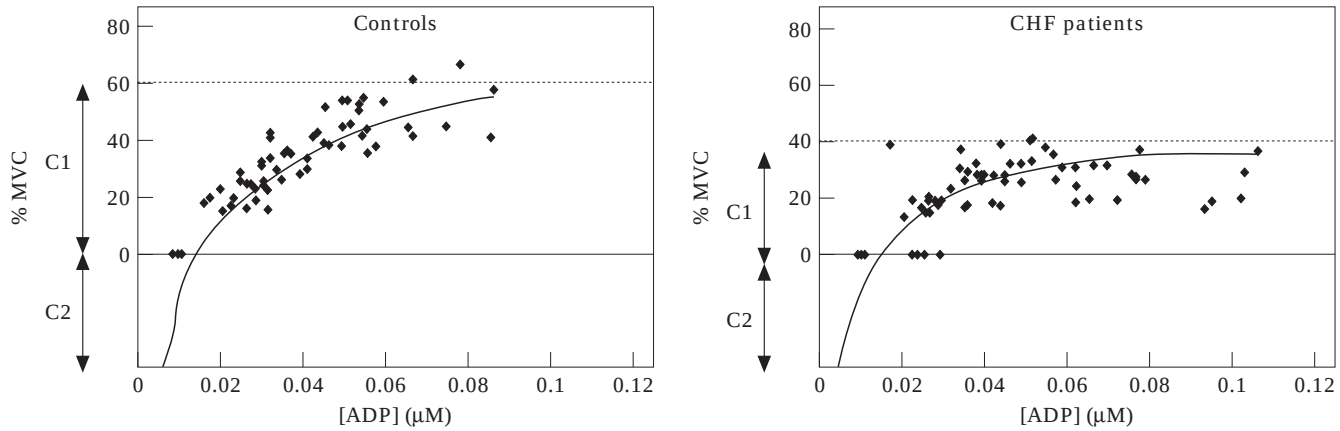

Figure 3 **Graph showing the hyperbolic relationship between intracellular ADP concentrations and maximum voluntary contraction (MVC). In CHF patients the C1 value, indicating maximal O_2 consumption, is significantly lower than in normals. The basal O_2 consumption however, is comparable in both groups. C1–C2, which represents the maximal ATP-fuelled steady-state power output, is significantly lower in CHF patients than in normals.**

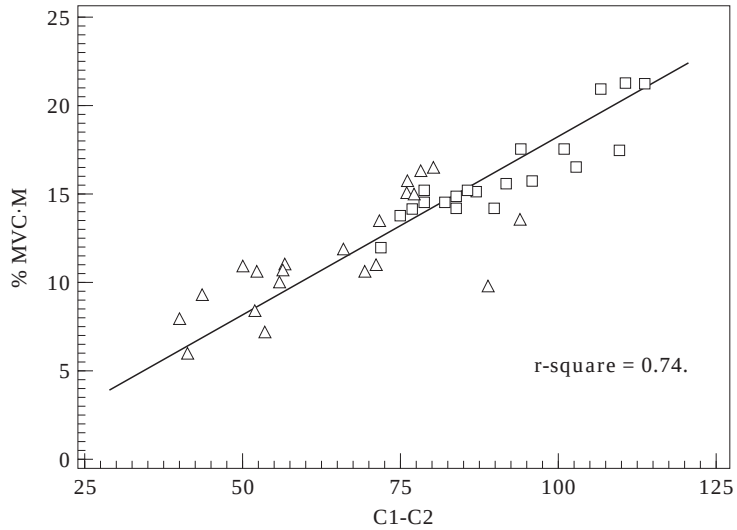

Figure 4 **Graph showing the linear correlation of phosphorylation rate m against the maximal steady-state power output C1-C2. Correlation between these parameters is highly significant. Along this line, there is a difference in distribution between heart failure patients and normal individuals. △ = chronic heart failure patients; □ = controls.**

exercise at normalized, submaximal workloads was strikingly homogeneous for the 22 patients. No patients exceeded the range of mean ± 2 standard deviations for these parameters. This observation is in sharp contrast with the findings in previously reported studies of forearm flexor muscle bioenergetics in heart failure patients, where a highly heterogeneous metabolic response to identical exercise regimens was observed amongst patients[4]. We attribute this homogeneity in our data to the adequate control of the population of fibres that was metabolically and mechanically sampled in the present study. This was also illustrated by the observation that in both patients and normal individuals at the highest workload the lowest-measured intracellular pH was 6·90, only 0·15 units lower than the resting state value (7·05) and homogeneous amongst fibres within the sampled muscle mass, as indicated by the narrow line width of the phosphate peak (Fig. 1). According to studies on the transition of aerobic to anaerobic metabolism in human forearm muscle employing both ^{31}P NMR and electromyography, this indicates that oxidative (type I and IIa), and not glycolytic (IIb) fibres were recruited during the voluntary exercise programme used[16,17]. In addition, the maximum workload in this study was at a very modest level (no more than 40% of maximum voluntary contraction). Thus, the maximum work demanded by the protocol was not limited by the number and oxidative capacity of mitochondria present in the recruited fibres. Therefore, the present protocol was not used to determine the exercise limit in these

Table 2 Individual values of metabolic parameters assessed by ^{31}P NMR spectroscopy of human forearm muscle

Heart failure patients				Normal individuals			
Patient number	Baseline Pi/PCr ratio	C1–C2	m	Patient number	Baseline Pi/PCr ratio	C1–C2	m
1	0·13	76	16	1	0·08	114	21
2	0·12	52	8	2	0·08	90	14
3	0·12	44	9	3	0·10	84	14
4	0·11	57	11	4	0·09	110	17
5	0·13	48	18	5	0·09	111	21
6	0·14	78	16	6	0·08	79	14
7	0·12	40	8	7	0·09	103	16
8	0·09	56	11	8	0·09	101	17
9	0·13	66	12	9	0·10	92	15
10	0·13	70	11	10	0·08	107	21
11	0·11	56	10	11	0·08	75	14
12	0·17	77	15	12	0·09	79	15
13	0·08	71	11	13	0·08	72	12
14	0·15	54	11	14	0·10	82	15
15	0·15	72	13	15	0·10	94	17
16	0·15	94	13	16	0·09	84	15
17	0·14	80	16	17	0·07	87	15
18	0·13	76	15	18	0·11	86	15
19	0·15	50	11	19	0·07	77	14
20	0·17	89	10	20	0·10	96	16
21	0·08	41	6				
22	0·10	54	7				
$\bar{x}$ ± SEM	0·13 ± 0·005	63 ± 3	11·7 ± 3·0	$\bar{x}$ ± SEM	0·09 ± 0·002	91 ± 3	15·8 ± 2·5

Average Pi/PCr ratios at rest differ significantly between patients and normal individuals. However, these values do not relate to the metabolic parameters of activated mitochondria during low-grade exercise. Impaired maximum phosphorylation rates (m) and maximum ATP-fuelled steady-state power output (C1–C2) can be associated with normal baseline Pi/PCr ratios and vice versa.

patients. In this protocol, work was only used to get the mitochondria out of their resting state. The prerequisite condition for valid interpretation of our results with respect to mitochondrial functionality, i.e. that the measurements have to be performed in a population of fibres that is relatively constant in its mitochondrial content in both patients and normal subjects, was therefore adequately met. This contrasts with previous studies and is likely to contribute to the reproducibility of the data obtained in our study. The use of a control group that was not age matched in this study is justified. An 'in vitro' study by Trounce *et al*. suggested that mitochondrial function declines with age[18], however this was most pronounced in the age group over 75, which had skeletal muscle respiration rates of about half the rate in younger subjects. In our study, respiration rates were reduced by about 50% independent of age, whereas all our patients were younger than 75 years. Moreover, previous studies failed to observe age-related differences in skeletal muscle bioenergetics, using nuclear magnetic resonance[19]. This suggests that there were skeletal muscle abnormalities other than functional changes induced by age alone. Even so, the protocol used in our study does not depend on changes in the number of mitochondria or secondary muscular atrophy. This is accounted for by the use of a relatively low power output during exercise[10].

In a small subgroup of patients, a transient rapid decline of pH was observed during exercise at the first workload only, recovering to the range of normal values during exercise at subsequent workloads. Although local blood flow was not measured in this study, this may be interpreted as reflecting a temporal flow impairment causing rapid deoxygenation and increased anaerobic glycolytic flux. Apparently, spontaneous flow adaption occurred at subsequent workloads in view of the transient nature of the decline in pH. This phenomenon has also been observed in normal subjects, albeit less commonly[20]. Since this was only observed in a small subgroup of patients, the results of the present study can be considered supportive of the hypothesis that flow impairment is not a major contributor to the abnormalities of skeletal muscle bioenergetics in heart failure[4,6,21].

In patients, abnormal baseline phosphate/phosphocreatine ratios were observed in the presence of normal baseline phosphorylation rates. In addition, basal phosphorylation rates and oxygen consumption were comparable in patients and normal subjects. However, the lower phosphorylation rates during exercise, indicated by a lower value for slope **m** and intercept **b** (Fig. 2), demonstrate that the intracellular energy buffer is depleted more rapidly in patients than in normal individuals. This is confirmed by the lower maximal O_2

consumption (C1) and lower maximal steady-state power output (C1–C2), reflected by more ADP production at lower power output levels. Under conditions of adequate flow and homogeneous, muscle fibre activation, this observation is in agreement with previous studies of skeletal muscle energy metabolism[2,3,7]. Also, the prolonged recovery after exercise is in line with a reduced capacity for oxidative phosphorylation.

In the present study, skeletal muscle mitochondrial dysfunction was present and is most likely involved in the clinical status of our heart failure patients. 'In vivo' oxidative phosphorylation capacity parameters were all reduced to the same extent, i.e. approximately by 30%. In a plotted representation of maximal phosphorylation rates and maximum steady-state power output, reflecting mitochondrial function (Fig. 4), heart failure patients can be distinguished from normal subjects. The protocol used provides us with a non-invasive means of collecting 'in vivo' data, which are highly reproducible. Whether these values are related to the severity or duration of heart failure cannot be deduced from our study results.

In conclusion, in patients with chronic heart failure, aerobic metabolism in peripheral muscle becomes already impaired at very modest exercise levels under conditions where acidosis is avoided and predominantly oxidative phosphorylation pathways are activated. Therefore, the cellular capacity for oxidative phosphorylation is substantially reduced in these patients compared to normal individuals.

In addition to the established lower mitochondrial density of skeletal muscle in heart failure, mitochondrial dysfunction may contribute to the clinical feature of reduced exercise tolerance. The underlying mechanisms are not entirely clear. Abnormal skeletal muscle flow may be one cause, although in our study abnormalities in aerobic metabolism already occurred under conditions in which flow may be presumed to be adequate. Deconditioning could be another reason. Several studies suggest that exercise training may improve skeletal muscle metabolism in heart failure patients, although these studies do not focus on aerobic metabolism, as was done in the present study[22,23].

The observation of intrinsic abnormalities in oxidative phosphorylation in the present study supports the potential usefulness of metabolic therapy aimed at improving aerobic muscle metabolism. This novel form of therapy is rapidly gaining interest in the management of heart failure.

Note added in proof

It has since been learned that equation (3) that was used to estimate the metabolic parameter C1-C2 is second, not first order in [ADP] (Jeneson JAL, Wiseman RW, Westerhoff HV, Kushmerick MJ: The signal transduction function for oxidative phosphorylation is at least second order in ADP. J Biol Chem, 1996, 271: 27995–27998). While this affects the *absolute* value of C1-C2 that is obtained from fitting eqn (3) to the ([ADP], power-output) data, this would not alter the *relative* change in C1-C2 in patients versus controls that was studied here (Fig. 4).

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