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Cytochrome *c* Oxidase and Purine Nucleotides in Skeletal Muscle in Tumour-bearing Exercising Rats

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We have previously shown that spontaneous physical exercise can delay the onset of experimental anorexia and cachexia and retard tumour growth and we now report the effects on the energy metabolism in skeletal muscle. Exercising tumour-bearing animals (TBE) had an increased maximal capacity for oxygen uptake expressed as V_{max} of the cytochrome *c* oxidase compared with their tumour-bearing sedentary controls (TBS) [mean (S.E.) 289.9 (30.7) vs. 141.6 (11.0); $P < 0.05$] but an unchanged K_m value. The TBS animals had a depressed V_{max} as compared with non-tumour-bearing sedentary controls (CS) [141.6 (11.0) vs. 210.1 (15.1); $P < 0.05$]. Most of the purine nucleotides in the 'glycolytic' anterior tibial muscle were significantly altered in the TBE animals compared with the TBS animals, but in the mainly 'oxidative' soleus muscle only the level of inosine monophosphate (IMP) was changed. The results indicate that physical exercise can normalise the oxidative capacity and improve the energy state in skeletal muscle in the tumour-bearing host.
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INTRODUCTION

IT HAS long been known that endurance training increases the oxidative capacity in skeletal muscle [1, 2]. It is also known that skeletal muscle metabolism is greatly altered in response to cancer anorexia and cachexia [3, 4]. Less known, however, is the effect of spontaneous physical exercise on the oxidative capacity in skeletal muscle in the tumour-bearing host. We have shown that the turnover number of cytochrome *c* oxidase (E.C.1.9.3.1), the terminal component of the mitochondrial respiratory chain, is increased in rats subjected to standardised physical exercise [5], and reduced in ischaemic and reperfused skeletal muscle tissue [6]. More recently, we have shown that spontaneous physical exercise in an experimental model with tumour-bearing rats could delay the onset of anorexia and cachexia and retard tumour growth [7]. This animal model is in contrast with most of the earlier work in the field with semi-voluntary or forced physical exercise in order to standardise the procedure [7].

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The abnormality in the energy metabolism of the tumour-bearing host is characterised by an increase in energy expenditure [8–10] that results in a cumulative negative energy balance with energy depletion. The adverse effects of a reduced caloric intake are added to this metabolic derangement. Provision of extra calories in order to modify the derangement is often unsuccessful and might even result in adverse effects on the tumour host metabolism [11, 12] as well as an increase in the tumour burden [13]. The peripheral metabolic alterations that facilitate gluconeogenesis tend to parallel the increased tumour burden.

The finding that tumour-bearing animals increased their food intake in response to physical exercise without increased tumour growth and with preserved skeletal muscle mass raised the question whether this exercise could normalise tumour induced changes in oxidative capacity and energy metabolism in skeletal muscle. In this paper we present a kinetic analysis of the cytochrome *c* oxidase reaction and an evaluation of the purine nucleotides in this experimental model with freely-moving tumour-bearing rats.

MATERIALS AND METHODS

Animal and tumour model

Female Wistar Furth rats were used. All experiments were performed in growing animals and they were allocated to the

study groups and to the corresponding control groups matched for body weights. A total number of 40 animals were divided into four equal groups: tumour-bearing exercising animals (TBE), tumour-bearing sedentary animals (TBS), non-tumour-bearing exercising animals (CE) and non tumour-bearing sedentary animals (CS). The animals in each tumour group were implanted with a transplantable Leydig cell tumour (LTW). This tumour neither metastasises nor penetrates into the adjacent tissues, it is well defined and has been described elsewhere [14]. The Leydig cell tumour has been kept in passages in our laboratory for 9 years without any changes in histological findings.

After adaptation to the cages, implantation or sham operation was performed under ether anaesthesia using a trocar. The animals in each tumour group were implanted with 1.5 mm^3 of viable tumour tissue subcutaneously in each flank, while the control animals received the same volume of 0.9% NaCl solution. All the animals were killed on day 32 after tumour implantation or sham operation. The animals allocated to exercise were individually housed in cages open to the interior of a freely-moving, wire-bottomed and non-motorised running wheel with a diameter of 33 cm (UNO Roestvaststaal BV, Arnhem, Holland). These animals had free access to the running wheel throughout the experiment. The other half of the animals were individually housed in standard cages. All animals had free access to a balanced diet (EWOS—ALAB brood stock feed for rats and mice, ALAB, Sollentuna, Sweden) and tap water. Room temperature was kept at [mean (S.E.)] 22 (1°C) and the light:dark cycle was 12:12 h. Body weight and food intake were recorded every fourth day while distance run was recorded daily. The experimental model was approved by the Ethical Committee of the University of Göteborg, Sweden.

Sample collection

On day 32 after tumour implantation or sham operation, all animals were killed. They were anaesthetised with sodium pentobarbital (ACO, Solna, Sweden) intraperitoneally (50 mg/kg body weight). Skeletal muscle samples were taken from both hind limbs: from one calf, a cross-section biopsy was taken for immediate homogenisation and from the other calf, the soleus and anterior tibial muscles were dissected, removed and immediately frozen in liquid N_2. The tumours were dissected free and weighed. Two biopsy samples from each tumour were dried at 80°C to constant weight. Finally, the animals were killed by bleeding.

Chemicals

Horse heart cytochrome *c* (type VI), *N,N,N',N'*-tetramethyl-paraphenylenedihydrochloride (TMPD), L-ascorbic acid (Na salt), nucleotides, nucleosides and purine bases were purchased from the Sigma Chemical Co. All other chemicals used were of analytical grade.

Cytochrome c oxidase kinetics

Homogenates for analysis of cytochrome *c* oxidase kinetics were prepared from fresh calf muscle tissue (soleus, tibial, plantar, gastrocnemius and EDL) [5]. The steady-state reaction of cytochrome *c* with cytochrome *c* oxidase was followed polarographically in a 50 mmol/l potassium phosphate medium, pH 7.5 at 25°C in the presence of ascorbate and TMPD as reducing agents with a Clark oxygen electrode as described by Soussi *et al.* [5]. The activity was determined at six consecutive cytochrome *c* concentrations (12.50–187.50 μmol/l). Initial reaction velocities (V_o) were expressed as μmol of oxygen consumed per min per gram protein. The maximal velocity (V_{max}) and the Michaelis constant (K_m) were calculated from Hanes plots ([cytochrome *c*]/V_o vs. [cytochrome *c*]).

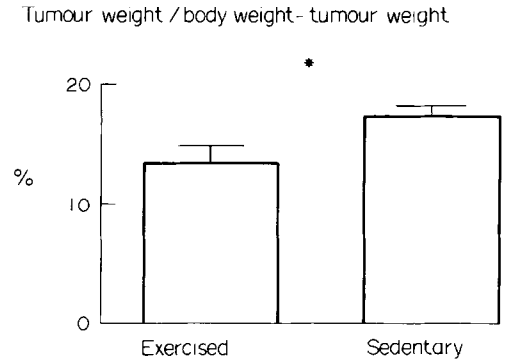

Fig. 1. Tumour weight relative to body weight (weight/total body weight – tumour weight). $n = 10$ in each group.

HPLC analysis

Biopsies were taken from the anterior tibial muscle, which is mainly composed of fast twitch glycolytic fibers and from the soleus muscle, mainly consisting of slow twitch oxidative fibers. Samples were frozen in liquid nitrogen, freeze-dried, minced to powder and extracted with 1.5 mmol/l perchloric acid solution containing 1 mmol/l EDTA. The neutralised extracts were injected into a high performance liquid chromatography system (Pharmacia AB, Sweden). The separation was performed on a C2/C18 silica, Mino RPC S 5/20 column and all purine nucleotides in the sequence from ATP to uric acid were determined in a single run as described in detail elsewhere [15]. The concentrations of ATP, ADP, AMP, IMP, adenosine, inosine, hypoxanthine plus xanthine and uric acid were calculated from the computer-integrated areas of the sample, relative to the areas obtained for the standard solutions. The energy charge was calculated according to Atkinson from the equation:

$$EC = [ATP] + 1/2\,[ADP]/[ATP] + [ADP] + [AMP].$$

Statistics

The values are presented as the mean and standard error of the mean [mean (S.E.)]. Statistical significance was tested with the Mann–Whitney U-test and a *P*-value of < 0.05 was considered significant.

RESULTS

Body weight, food intake and physical activity

The results are in good agreement with those presented in the methodological article on this experimental model (cf. Ref. 7).

In short, there were no differences between the TBE and the TBS animals concerning total body weight throughout the experiment. Both groups of exercising animals had increased food intake compared with their sedentary controls after the 4th day of the experiment. The TBE animals had increased food intake compared with the TBS animals until day 28 or 32, i.e. until the onset of late cachexia. As to the daily distance run there was a significant difference between the two exercising groups from day 20 and onwards. The TBE group showed a gradual decrease in running distance per day but preserved an ability for physical exercise despite a growing tumour burden.

Tumour weight

Lower relative tumour weights were recorded in the exercising group (TBE 13.4 (1.4)%, TBS 17.4 (0.8); $P < 0.05$) (Fig. 1).

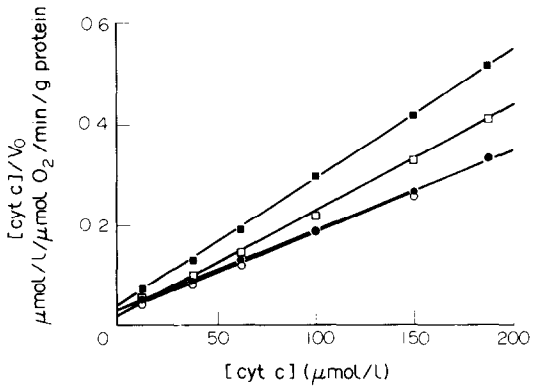

Fig. 2. Hanes plots showing the effect of physical exercise on the kinetic parameters V_{max} and K_m of cytochrome c oxidase in rat skeletal muscle. Each line represents the mean value of each group and the correlation coefficient is 0.998 (cf. Table 1). TBE ●; CE ○; TBS ■; CS □.

The water content of the tumours did not differ between the groups (TBE 83.1%, TBS 83.3%; N.S.).

Cytochrome c oxidase kinetics

The initial velocity of the cytochrome *c* oxidase reaction was determined in muscle homogenate at different substrate concentrations (cytochrome *c*) ranging from 12.50 to 187.50 μmol/l at an ionic strength still allowing biphasic kinetic behavior at the chosen pH [16]. Hanes plots ([cytochrome *c*]/V_0, against [cytochrome *c*]) were constructed for each muscle homogenate. The maximal velocity of the enzyme reaction (V_{max}) was calculated from the slope of the line (correlation coefficients, $r > 0.998$) and the Michaelis constant (K_m) was represented by the *X*-intercept. These plots are summarised in Fig. 2, where each line is the mean value of each group.

The kinetic parameters for cytochrome *c* oxidase in skeletal muscle of each animal in the four groups (TBE, TBS, CE and CS) are presented in Table 1. The variations between the animals are inter individual variations as evidenced by the high *r*-values of the linear regression analysis of each individual Hanes plot. It is shown that physical exercise in the control animals (CE) enhances the V_{max} by more than 28% ($P < 0.05$) when compared with their sedentary control group (CS). On the other hand, the V_{max} in the tumour-bearing sedentary rats (TBS) is 33% ($P < 0.05$) lower than in the sedentary controls (CS). However, the V_{max} in the TBE-group is doubled ($P < 0.05$) as a result of exercise. The reduced oxidative capacity in the TBS group is thus raised above the CS value to become compatible with the trained normal controls (CE). The K_m of cytochrome *c* oxidase in rat skeletal muscle remained approximately the same in all groups, showing that the affinity of the enzyme to its natural substrate is unchanged.

In the sedentary tumour-bearing animals (TBS), there were significant negative correlations between V_{max} and tumour wet weight ($r = -0.97$; $P < 0.01$) and relative tumour weight ($r = -0.95$; $P < 0.01$), respectively. No such correlations were found in the tumour-bearing exercising animals (TBE) but instead a positive correlation was found between V_{max} and the total body weight gain during the experiment ($r = 0.84$; $P < 0.05$).

Purine nucleotides

The concentrations of ATP, ADP, AMP, IMP, inosine, adenosine, hypoxanthine plus xanthine, uric acid and the energy charge in the tibial and soleus muscles of the four groups investigated are presented in Tables 2 and 3. In the anterior tibial muscle, which is mainly composed of fast twitch glycolytic fibres, there were significant differences between the trained groups and their respective sedentary control groups concerning most of the nucleotides (Table 2). No such differences were found between the two groups subjected to exercise or between the two sedentary groups. The pattern concerning the nucleotide concentrations in the soleus muscle, composed mainly of slow twitch oxidative fibres, showed fewer differences between the groups (Table 3). The concentration of IMP in the soleus muscle in the TBE animals was, however, decreased compared to their sedentary controls (TBS). The energy charge in the anterior tibial muscle in the TBE animals was significantly increased compared with their sedentary controls and this was even significantly increased compared with the normal sedentary controls (CS). These differences were not found in the soleus muscle. The ATP concentration in the anterior tibial muscle as compared with that in the soleus muscle was increased in both the exercising groups (TBE $P < 0.01$; CE $P < 0.05$) but this difference between skeletal muscles of different types was not found in any of the sedentary groups.

Table 1. Kinetic parameters of cytochrome c oxidase in calf muscle in tumour-bearing exercising (TBE), tumour-bearing sedentary (TBS), control exercising (CE) and control sedentary animals (CS)

Animal	K_m (μmol/l)	V_{max} (μmol O_2/min × g protein)
TBE 1	11.6	264.4
TBE 2	10.6	238.5
TBE 3	21.3	260.7
TBE 4	18.0	410.4
TBE 5	12.2	275.4
Mean (S.E.)	14.7 (2.1)	289.9 (30.7)
TBS 1	11.1	154.9
TBS 2	13.5	110.1
TBS 3	16.1	158.1
TBS 4	10.9	143.4
Mean (S.E.)	12.9 (1.2)	141.6 (11.0)
CE 1	15.6	269.6
CE 2	6.1	219.6
CE 3	11.3	250.4
CE 4	10.1	336.5
CE 5	12.1	274.4
Mean (S.E.)	11.0 (1.5)	270.1 (19.2)
CS 1	11.4	189.5
CS 2	4.0	193.4
CS 3	8.3	179.0
CS 4	11.4	227.4
CS 5	18.7	261.1
Mean (S.E.)	10.8 (2.4)	210.1 (15.1)

V_{max} $P < 0.05$: TBE vs. TBS, TBS vs. CS, TBS vs. CE, CE vs. CS. Each K_m and V_{max} value is derived from six activity measurements at different cytochrome *c* concentrations (12.50–187.50 μmol/l); Hanes plots were used ($r = 0.998$). Values are means (S.E.).

Table 2. The intramuscular concentration (μmol/g dry weight) of various nucleotides and the energy charge in the anterior tibial muscle in tumour-bearing exercising (TBE), tumour-bearing sedentary (TBS), control exercising (CE) and control sedentary (CS) animals

	TBE	TBS	CE	CS
IMP	1.09 (0.03)*	1.22 (0.03)	1.10 (0.07)	1.14 (0.08)
ATP	33.38 (2.01)*	26.08 (1.84)	34.27 (1.82)†	30.06 (0.71)
ADP	4.86 (0.63)*	7.19 (0.24)	4.67 (0.88)†	7.45 (0.27)
HX + X	0.69 (0.06)	0.97 (0.24)	0.83 (0.08)†	1.09 (0.08)
AMP	0.58 (0.06)*	0.91 (0.07)	0.73 (0.11)	0.87 (0.14)
Ino	6.32 (0.28)*	5.55 (0.11)	6.39 (0.28)†	5.36 (0.08)
UA	< 0.2	< 0.2	< 0.2	< 0.2
EC	0.922 (0.010)*‡	0.867 (0.007)	0.923 (0.011)†	0.880 (0.005)

* $P < 0.05$, TBE vs. TBS; † $P < 0.05$, CE vs. CS; ‡ $P < 0.05$, TBE vs. CS.
Values are means (S.E.). $n = 5$ in each group.
Ino = inosine; HX + X = hypoxanthine + xanthine; UA = uric acid; EC = energy charge.

Table 3. The intramuscular concentration (μmol/g dry weight) of various nucleotides and the energy charge in the soleus muscle in tumour-bearing exercising (TBE), tumour-bearing sedentary (TBS), control exercising (CE) and control sedentary (CS) animals

	TBE	TBS	CE	CS
IMP	0.94 (0.05)*	1.16 (0.03)	1.05 (0.08)	1.10 (0.09)
ATP	24.70 (1.28)	23.09 (1.45)	29.82 (0.66)	29.71 (1.03)
ADP	5.99 (0.21)	6.45 (0.11)	6.15 (0.25)	6.71 (0.24)
HX + X	0.50 (0.13)	0.56 (0.23)	0.60 (0.06)†	1.00 (0.07)
AMP	0.72 (0.20)	0.86 (0.11)	0.46 (0.03)†	0.80 (0.07)
Ino	5.45 (0.25)	5.34 (0.19)	5.46 (0.04)	5.67 (0.16)
UA	< 0.2	< 0.2	< 0.2	< 0.2
EC	0.882 (0.007)‡	0.865 (0.007)§	0.903 (0.003)†	0.888 (0.005)

* $P < 0.05$, TBE vs. TBS; † $P < 0.05$, CE vs. CS; ‡ $P < 0.05$, TBE vs. CE; § $P < 0.05$, TBS vs. CS.
Values are means (S.E.). $n = 5$ in each group.
Ino = inosine; HX + X = hypoxanthine + xanthine; UA = uric acid; EC = energy charge.

DISCUSSION

In this paper we report a kinetic investigation of cytochrome *c* oxidase in skeletal muscle in the tumour-bearing rat during spontaneous physical exercise. The maximal capacity for oxygen uptake is expressed as the V_{max} of the oxidase, since common activity measurements (V_o) at a single substrate concentration are insufficient and can be misleading due to the complexity of this complex IV of the mitochondrial respiratory chain [5].

This animal model is different from most of the previous studies in the field, where different kinds of semi-voluntary or forced exercise have been used in order to standardise the experimental procedure [7]. Spontaneous physical exercise could reduce the neuroendocrine response associated with stress and thus make it possible to delineate the beneficial effects of physical exercise. Our findings are in general agreement with those reported by K.J. Rodnick *et al.* [17].

We have recently demonstrated that it is possible to delay the onset of cancer anorexia and cachexia and retard tumour growth in spontaneously running tumour-bearing rats [7]. The tumour-bearing animals increased their food intake compared with their sedentary controls early in the experiment but despite these extra calories tumour growth was retarded and the final relative tumour weights were lower. The exercising animals were able to better preserve their body composition regarding total body protein and the RNA/protein quotient in skeletal muscle was increased. In spite of late cachexia, most animals were able to run 2–5 km a day. The changes in energy metabolism associated with cancer cachexia include an increased rate of endogenous glucose production which has been shown in both rodents and man and this increase is correlated to tumour stage [18, 19]. Several studies have shown an increase in the rate of the Cori cycle [20–23] but the role played by this futile energy cycling is still controversial. The increased rate of glucose oxidation does not seem to parallel the glucose availability, thus indicating a deficiency of the oxidative process [19]. Activities of enzymes in the oxidative metabolism in skeletal muscle from cancer patients [3] and rodents [3, 24] have been found to be reduced. In a recent study by Schneeberger *et al.* [25], skeletal muscle metabolism in tumour-bearing rats was studied with ^{31}P-NMR and the P_i/ATP ratio was found to be increased with increasing tumour burden. These combined findings indicate a peripheral energy depletion and a reduced oxidative capacity in order to facilitate gluconeogenesis. As these changes tend to parallel the increased tumour burden and part of the glucose is used as energy substrate by the tumour, we found it important to study if physical exercise could normalise the metabolic pattern in skeletal muscle in the tumour-bearing animal.

Cytochrome *c* oxidase is a multi-function enzyme that spans the inner mitochondrial membrane. It couples electron transfer between cytochrome *c* and molecular oxygen, driving ATP synthesis, which warrants a detailed kinetic study and an investigation of the high-energy phosphate metabolism.

The present study shows that the oxidative capacity, expressed as the V_{max} of cytochrome *c* oxidase, is reduced in the tumour-bearing sedentary animals. This is also accompanied by a lower ATP concentration than in sedentary controls, indicating a lower oxygen uptake, a reduced mitochondrial oxidative phosphorylation and a slower ATP synthesis. On the other hand, exercise not only increases the V_{max} of the enzyme as has been shown previously [5] but also ameliorates the V_{max} of the tumour-bearing rats to a degree reaching the value of the exercising normal control animals. Failure of mitochondrial respiratory components in skeletal muscle has been shown earlier to occur as a consequence of various metabolic disorders. We have earlier interpreted the V_{max} decrease in skeletal muscle subjected to reduced blood flow and subsequent reperfusion in terms of altered mitochondrial membrane structure and function [6]. Hence, the low V_{max} in the tumour-bearing sedentary animals could be due to lower oxygen uptake and/or uncoupled mitochondria. Support for this explanation comes from a structural ^{1}H-NMR investigation of the interaction between cytochrome *c* and cardiolipin demonstrating the absolute requirement of cardiolipin for the binding of the protein to it and for optimal cytochrome *c* oxidase activity [26].

The analysis of two different types of skeletal muscle shows that the patterns of purine nucleotides are different in the anterior tibial muscle (mainly fast twitch glycolytic fibres) compared with the soleus muscle (mainly slow twitch oxidative fibres). In the anterior tibial muscle, significant differences are shown for most of the nucleotides in both the exercising groups compared with their sedentary controls while no differences are

shown between the exercising groups and the sedentary control groups in the soleus muscle. Furthermore, the concentration of ATP in the anterior tibial muscle compared with that in the soleus muscle shows significantly increased values in both the exercising groups but not in the sedentary groups. These findings indicate a real effect of the training regimen. On the other hand, this altered pattern of the purine nucleotide concentrations was not found in the soleus muscle, probably due to the different fibre composition. The concentration of IMP in the soleus muscle was significantly increased in the tumour-bearing sedentary animals compared to the tumour-bearing animals subjected to exercise. This increase might be coupled to the altered energy state and the increased V_{max} associated with an increased dephosphorylation of IMP. The pattern of the purine nucleotides in the anterior tibial muscle, combined with the increased energy charge, indicates that the training regimen in this experimental model mostly affects the fast twitch glycolytic skeletal muscle fibers. The energy cost in the fast-twitch fibre type is higher than in the slow-twitch fibre [27]. It has also been shown that the activity of cytochrome oxidase is higher in the slow twitch muscle than in the fast twitch one [28]. Accordingly, the tibial muscle was found to be energetically more sensitive to ischemia than the soleus muscle [15]. Apart from the functional differences between the two muscle types, the difference in intracellular buffer capacity could be a factor contributing to its response to energy metabolic changes.

This study shows that it is possible, by means of spontaneous physical exercise, to improve the altered oxidative capacity and energy charge in skeletal muscle in the tumour-bearing host. As we have reported a delayed onset of cachexia in the tumour-bearing animal subjected to physical exercise with a better preserved skeletal muscle mass [7], the combined results indicate an improvement in skeletal muscle function. As the tumour weights were lower despite an early increase in food intake, these results in an experimental setting indicate a possibility of improving the anorectic and cachectic state in the cancer patient.

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