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Biochimica et Biophysica Acta 1276 (1996) 45–50

BBA Biochimica et Biophysica Acta

Proton leak and control of oxidative phosphorylation in perfused, resting rat skeletal muscle

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Received 9 February 1996; revised 20 March 1996; accepted 21 March 1996

Abstract

The distribution of control over the resting respiration rate (and other variables) was assessed in perfused rat skeletal muscle. The results indicate that the general pattern of control in resting muscle is similar to that seen in isolated rat liver cells (Brown et al. (1990) Biochem. J. 192, 355–362). Control over resting mitochondrial oxygen consumption was distributed between reactions involved in substrate oxidation (flux control coefficient = 0.44 ± 0.28) and those involved in ATP turnover (0.21 ± 0.09). The mitochondrial proton leak also had an important share of the control (0.38 ± 0.21). Since proton leak also has significant control over the resting respiration rate of isolated liver cells and liver and skeletal muscle account for around 60% of the standard metabolic rate of a rat, then these results indicate the potential importance of proton leak as a mechanism of regulating metabolic rate.

Keywords: Proton leak; Perfused muscle; Metabolic control

1. Introduction

The activity of the proton leak pathway is very different in mitochondria isolated from animals of different body mass [1] and phylogeny [2]. In general, mitochondria from smaller animals and endotherms have greater proton leak activity than those from larger animals and ectotherms. However, in hepatocytes isolated from these organisms, the proportion of the resting respiration rate used to drive the proton leak is the same in all cases [2,3]. The fact that these animals appear to maintain the balance between proton leak activity and that of other cellular processes may indicate an important role for proton leak in the 'short term' control of metabolic rate and thermogenesis.

Clearly, for this to be true, proton leak must have a high flux control coefficient over the resting metabolic rate of an animal. The fact that proton leak makes a significant *contribution to* Standard Metabolic Rate [4] does not mean that this process has significant *control over* SMR, because it is possible that increase in the activity of the proton leak pathway may cause a compensating decrease in activity of other pathways (e.g., ATP synthesis) which compete for the same intermediate metabolite. However, it has been shown that proton leak has significant control over the resting respiration rate of isolated rat liver cells (reviewed in Ref. [5]). The general pattern of control in these cells is similar to that seen in isolated rat liver mitochondria [6–8]. The pattern of control in mitochondria isolated from the major oxygen consuming organs of the rat (kidney, brain and skeletal muscle) is similar to that seen in isolated liver mitochondria [6]. It is therefore possible that the proton leak is an important controller of the standard metabolic rate of a rat. Also, since oxygen consumption in the standard state is equivalent to heat production, mitochondrial proton leak may be an important thermoregulatory mechanism. Note here that throughout this paper, proton leak will be assumed to be the sole pathway responsible for mitochondrial non-phosphorylating respiration, a position for which ample evidence exists [9]. Other pathways, such as 'slip' in the proton pumps of the respiratory chain [10] may also occur but their potential presence or absence will not alter any of the conclusions presented in this paper, assuming that slip is uniquely related to mitochondrial Δp.

This paper assesses the control over the resting oxygen

Abbreviations: BSA, bovine serum albumin; DMO, dimethyloxazolidine-2,4,-dione; SMR, standard metabolic rate; State 4, the state attained by isolated mitochondria under conditions which ensure a zero rate of oxidative ATP synthesis; TPMP, methyltriphenylphosphonium cation; $\Delta\Psi$, membrane potential; Δp, electrochemical proton gradient; ΔpH, pH gradient.

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PII S0005-2728(96)00029-1

consumption rate of perfused rat skeletal muscle. Skeletal muscle was chosen as a suitable model for this investigation since it is the single most important contributor to rat standard metabolism, accounting for 33–40% of SMR [11,12]. Liver accounts for 10–20% of SMR [11,13] so together these organs account for between 43 and 60% of rat SMR [11–13], other organs only accounting for a further 20% in total [11]. Furthermore, skeletal muscle has been proposed to be an important site of heat generation during cold-induced thermogenesis [14,15]. Thus, a knowledge of the control exerted by proton leak over resting skeletal muscle respiration would indicate the potential significance of proton leak as a regulator of rat SMR, when combined with the data already obtained for liver cells [5].

The isolated, perfused rat hindquarter was chosen as a suitable system in which to study skeletal muscle metabolism. The hindquarter represents up to 50% of the total skeletal muscle mass of a rat (calculated from Ref. [11]) and comprises a wide range of fibre types. Over 95% of the oxygen consumption rate of the hindquarter is due to skeletal muscle metabolism [16]. The hindquarter is therefore considered to be reasonably representative of the entire skeletal muscle mass of a rat.

Control in resting skeletal muscle was analysed using the top-down approach to metabolic control analysis. This involves conceptually dividing the system into blocks of reactions linked by one (or more) intermediate metabolite(s) and assessing the response of each of the blocks to small changes in the levels of the intermediate metabolites. The theory of metabolic control analysis and the details of the top-down approach are given in [7,17–19].

2. Materials and methods

2.1. Materials

All materials were obtained from Sigma Chemical Co. Fancy Rd., Poole, Dorset, except for TPMP-bromide and tetraphenylboron (Aldrich Chemical Co., Gillingham, Dorset), adenine nucleotides (Boehringer Mannheim (UK), Lewes, East Sussex), pentobarbitone (RBM Animal Health Ltd., Dagenham, UK) and radioisotopes (Dupont Radiochemicals).

2.2. Methods

2.2.1. Isolation and perfusion of the rat hindquarter

All experiments were performed using 2- to 3-month-old female Wistar rats weighing 220–270 g. They were maintained at a thermoneutral temperature, provided with access ad libitum to a complete diet (Rat and Mouse No. 1 (modified); SDS, 1 Stepfield, Witham, Essex) and drinking water. They were anaesthetised using 60 mg sodium pentobarbitone per kg body weight and transferred to a heated operating platform. Note that, assuming that pentobarbitone distributes evenly throughout the rat, this concentration of anaesthetic has no effect on the proton leak activity of isolated skeletal muscle mitochondria [4]. The hindquarter was then isolated essentially according to the method of Ruderman et al. [16], the procedure taking approx. 20 min up to the time of cannulation of the aorta and vena cava. The hindquarter was then perfused (using a closed, recirculating system) with 250 ml Krebs-Henseleit bicarbonate buffer (+calcium chloride; 2.5 mM) (pH 7.35), 37°C, gassed with 95% O_2/5% CO_2 and containing glucose (10 mM), lactate (10 mM), pyruvate (1 mM), defatted BSA (4 g/100 ml) and TPMP (1 μM), according to the protocol outlined in Ref. [4]. Note that the liver, heart, lungs, kidneys and intestines were cut out and discarded to ensure that they did not contribute to the subsequent measurements of tissue oxygen consumption and membrane potential. Once the perfusion had begun, the hindquarter was immersed in a physiological saline solution which was maintained at 35°C. The weight of tissue perfused was assessed using trypan blue as outlined in Ref. [4]. The weight of tissue perfused was in the range 24–27 g for all preparations. The pattern and extent of staining did not alter during the 2-h perfusion [4].

2.2.2. Measurement of oxygen consumption and membrane potential

Arterial and venous oxygen concentrations were measured using an oxygen electrode (Rank Bros., Bottisham, Cambridge, UK) and the oxygen consumption rate of the hindquarter was calculated as outlined in [4]. The mitochondrial membrane potential in situ was assessed using the lipophilic cation TPMP. The distribution of this probe was monitored either using a TPMP-sensitive electrode [20] in the perfusion circuit or using radioisotopes as outlined in Ref. [4].

2.2.3. Titrations of membrane potential and oxygen consumption

Once the perfused hindquarter had reached a steady state for oxygen consumption and TPMP uptake (see Ref. [4]), membrane potential and oxygen consumption were titrated as outlined in Refs. [4,7] and in the legend to Fig. 2 in order to determine the subsystem elasticities. The system under investigation is shown in Fig. 1. Basically, the method involves changing the activity of one branch of the system and measuring the response of the other branches to the new steady-state value of membrane potential thus produced. Oligomycin (which inhibits oxidative phosphorylation) was used to determine the elasticity of the substrate oxidation system to $\Delta\Psi$. Note that it was necessary to add dantrolene a few minutes before addition of oligomycin to the perfusate in order to protect against the secondary effects of oligomycin (see Ref. [4] for details). Titration of the oligomycin-inhibited state with small subsaturating aliquots of cyanide was used to determine the

elasticity of the proton leak to $\Delta\Psi$. Titration of the resting state (i.e., without oligomycin) with small sub-saturating aliquots of cyanide gave the elasticity of the proton leak plus the ATP turnover reactions to $\Delta\Psi$. The elasticity of the ATP turnover reactions alone were calculated by first subtracting the response of the proton leak flux to $\Delta\Psi$ [7,19].

2.2.4. Measurement of plasma membrane potential and ΔpH

Changes in plasma $\Delta\Psi$ were assessed via changes in perfusate potassium concentration and changes in mitochondrial ΔpH were measured using [^{14}C]DMO as outlined in Ref. [4].

3. Results and discussion

3.1. Effect of the experimental procedure on the steady-state rate of respiration and value of transmembrane electrochemical gradients in the perfused hindquarter

Each individual experiment lasted a maximum of 2 h. Experiments on 3 individual hindquarter preparations showed that during this time the resting oxygen consumption rate and total tissue DMO uptake (ΔpH) and TPMP uptake ($\Delta\Psi$) remained constant [4]. Furthermore, the steady-state value for oxygen consumption and membrane potential in hindquarters in which oxidative phosphorylation had been inhibited by oligomycin (+dantrolene) remained constant over the remainder of the experimental period if no further additions were made [4].

3.2. The suitability of $\Delta\Psi$ as an intermediate for top-down control analysis in perfused skeletal muscle

The validity of the top-down approach depends on the kinetics of the blocks being a unique function of the magnitude of the chosen intermediate metabolite or metabolites. The intermediate is the mitochondrial protonmotive force (Δp) and not the mitochondrial $\Delta\Psi$ as shown in Fig. 1. Thus the elasticities (calculated from the data of the type shown in Fig. 2 as a function of the total tissue $\Delta\Psi$) will not be directly related to the intermediate unless ΔpH is constant for all values of $\Delta\Psi$ within the experimental range. However, the muscle plasma membrane potential and ΔpH were unaffected by the experimental manipulation of mitochondrial respiration [4] in agreement with the data of Nobes et al. [21] who showed that plasma $\Delta\Psi$ and ΔpH of hepatocytes were not significantly affected by inhibition of mitochondrial respiration with oligomycin or with respiratory chain inhibitors. Hence the responses of the subsystem fluxes to $\Delta\Psi$ shown in Fig. 2 are directly related to the response of the subsystem fluxes to the true intermediate, Δp.

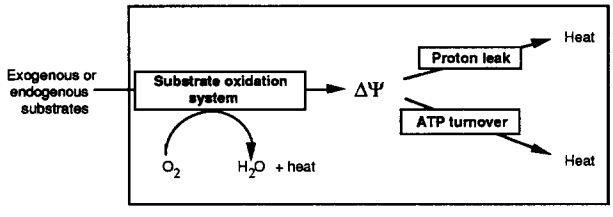

Fig. 1. Schematic representation of the metabolic system under consideration. 'The system' is everything within the box. $\Delta\Psi$ is produced by the 'substrate oxidation' subsystem, consisting of all processes from substrate supply to the tissue to $\Delta\Psi$, and consumed by the 'proton leak' (which includes cation cycles as well as any intrinsic leakiness of the mitochondrial inner membrane) and the 'ATP turnover' subsystem, which includes all steps involved in the production of ATP from $\Delta\Psi$, and its subsequent consumption to drive the production of cellular low entropy structures. This gives the three-block system whose overall control coefficients can be determined as described in the text. Note that all measurements are made in the resting state and thus all oxygen consumption is equivalent to heat production.

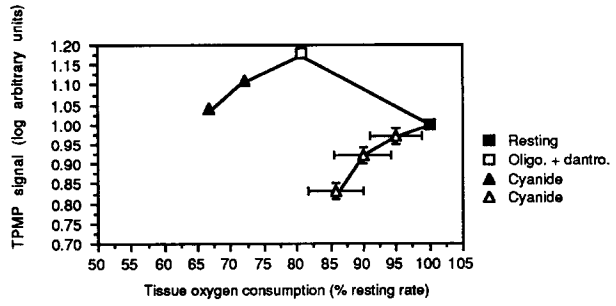

Fig. 2. The relationship between mitochondrial membrane potential and respiration rate in intact, perfused skeletal muscle. Data shown are from two types of experiments (experiments a and b) on individual hindquarters. Preparation and perfusion of each hindquarter was as described in Ref. [4] and in the text, but additions (after steady-state values for resting respiration and $\Delta\Psi$ were attained) were either (a) no additions (closed square), then dantrolene (7 μg/ml)+oligomycin (around 40 μg/g tissue) (open square) and finally progressive additions (around 20 μM) of sodium cyanide (closed triangles) or (b) progressive additions (around 20 μM) of sodium cyanide in the absence of other inhibitors (open triangles). Data shown here from experiment (b) represent the means ± S.E.M. of three independent experiments. The data were grouped in this way because the inhibitor concentrations used to obtain each datum point were identical in each experiment. This was not the case for experiments of type (a), and therefore the figure shows data from a single experiment of type (a) only. All the data from this figure, plus that from six further experiments of type (a), were used to calculate the system elasticities as explained in the text. Control coefficients (Table 1) were calculated from these elasticities according to Brand et al. [8,19] as explained in the text and in the legend to Table 1. Note that data for experiments of type (a) were taken from [4].

3.3. The assessment of non-mitochondrial respiration in the perfused hindquarter

In order to correctly calculate the control coefficients over oxidative phosphorylation, it was necessary to first subtract the non-mitochondrial respiration rate from each of the subsystem fluxes before the subsystem elasticities

were calculated (see below). The non-mitochondrial oxygen consumption rate was determined in the presence of excess (1–2 mM) sodium cyanide. In hepatocytes, the figure for non-mitochondrial oxygen consumption obtained using either sodium cyanide or myxothiazol was sufficiently similar to justify the assumption that 1–2 mM sodium cyanide had no effect on non-mitochondrial respiration [4].

3.4. Control of oxidative phosphorylation in resting skeletal muscle

Control of oxidative phosphorylation in the perfused hindquarter was analysed using the top-down approach of metabolic control analysis [5,19]. The system of interest was divided into producers and consumers of the intermediate ($\Delta\Psi$). The system pertaining to resting muscle is shown in Fig. 1. Control over oxidative phosphorylation was analysed in the non-phosphorylating ('state 4') and resting states. The control coefficients were calculated as explained in Refs. [7,8,19] except that relative values of $\Delta\Psi$ were used to calculate the sub-system elasticities. This has no effect on the values of the control coefficients calculated as long as absolute values for the system fluxes are known. The 3 fluxes – proton leak (J_L), ATP turnover (J_P) and substrate oxidation (J_S) – were calculated at any given value of $\Delta\Psi$ as (J_S = total cellular respiration rate − non-mitochondrial respiration rate); ($J_P = J_S - J_L$) and (J_L = the non-phosphorylating respiration rate at any given value of $\Delta\Psi$).

The degree of control exerted by any branch of our simplified system of oxidative phosphorylation is expressed as a coefficient. Control over pathway flux is expressed as a flux control coefficient. This is the fractional change in the flux through a given branch resulting from an infinitesimally small change in the activity of the same or another branch. This gives information about the relative importance, under a certain set of conditions, of each branch of the system in controlling pathway flux. Control over the pathway intermediate ($\Delta\Psi$) is expressed as a concentration control coefficient. This is the fractional change in the 'concentration' (i.e., magnitude) of $\Delta\Psi$ due to an infinitesimally small change in the activity of any one of the branches. This gives information on the homeostatic control of membrane potential. Control over the effective P/O ratio by any branch of the system is calculated as the difference in the control exerted by that branch over the respiration rate and the phosphorylating rate [8]. The effective P/O ratio is the mechanistic P/O ratio (calculated from H^+/O and H^+/ATP ratios) multiplied by the proportion of the total issue respiration used to drive the proton leak flux.

The control over oxidative phosphorylation is analysed in non-phosphorylating (state 4) and resting states. In state 4, the coefficient of control over the two pathway fluxes (respiration rate and proton leak) by the two branches of the system (the proton leak and the substrate oxidation system) has a value between zero and one, one being all the control. In the resting state, the coefficient of control over two of the three pathway fluxes (phosphorylation and proton leak) by two of the three branches of the system (the proton leak and ATP turnover) may also have a negative value. This is because the two branches compete with each other for the intermediate ($\Delta\Psi$), and increasing flux through one branch will result in a decrease in flux through the other. In both state 4 and the resting state, the sum of the control coefficients of the three branches over a given pathway flux is unity [17]. The sum of the control coefficients of the three branches over $\Delta\Psi$ and the P/O ratio is zero since all measurements are made in the steady state.

Table 1 shows the control coefficients calculated as outlined above. The data from which these coefficients were calculated are shown (in part) in Fig. 2, as explained in the legend to that figure and in the text. From Table 1 we can see that there is a significant amount of control invested in both the producers and the consumers of the intermediate ($\Delta\Psi$), in agreement with previous studies using isolated skeletal muscle mitochondria [6,22,23]. Thus, the main conclusion that can be drawn from this data is that the idea that muscle respiration is controlled solely by ATP demand (e.g., [24–28]) does not apply to resting skeletal muscle. The data show that it is possible to stimulate skeletal muscle respiration by increasing the activity of either the substrate oxidation system, the proton leak or the reactions involved in ATP turnover.

The overall control pattern is similar to that measured in hepatocytes [5,29], although there are some interesting differences. However, due to the large errors associated with the data from our analysis, these differences are statistically insignificant. Thus, the only conclusion that can be drawn from the data shown in Table 1 is that the distribution of control in perfused skeletal muscle and isolated liver cells is similar.

Concentrating on proton leak, we can see from Table 1 that in resting skeletal muscle the control over respiration rate by the leak (0.38 ± 0.21) is similar to that in resting liver cells (0.21–0.22; [5,29]). Thus it appears that proton leak has significant control over the rate of resting oxygen consumption (and therefore over the rate of heat production) in the two organs that account for the bulk of resting tissue oxygen consumption in the rat. Thus, proton leak has the potential to be an important thermoregulatory mechanism in mammals and therefore a target for hormones regulating facultative thermogenesis.

The results of this control analysis cannot be used to predict the effect on respiration of the large changes in proton leak that might be occurring during cold acclimatization, because the analysis only applies to infinitesimally small changes in conditions. Further work on this subject would involve an assessment of the contribution of proton leak to liver and muscle respiration in the cold acclimatised rat.

Table 1
Control of oxidative phosphorylation in perfused resting rat skeletal muscle

1. State 4:		2. Resting:	
Control over respiration:		Control over respiration:	
(i) by substrate oxidation	0.39 ± 0.25	(i) by substrate oxidation	0.44 ± 0.28
(ii) by proton leak	0.61 ± 0.25	(ii) by proton leak	0.38 ± 0.21
		(iii) by phosphorylation	0.21 ± 0.09
Control over proton leak:		Control over proton leak:	
(i) by substrate oxidation	0.39 ± 0.25	(i) by substrate oxidation	0.50 ± 0.40
(ii) by proton leak	0.61 ± 0.25	(ii) by proton leak	0.71 ± 0.18
		(iii) by phosphorylation	−0.20 ± 0.16
		Control over phosphorylation:	
		(i) by substrate oxidation	0.47 ± 0.19
		(ii) by proton leak	−0.31 ± 0.13
		(iii) by phosphorylation	0.82 ± 0.08
Control over $\Delta\Psi$:		Control over $\Delta\Psi$:	
(i) by substrate oxidation	0.34 ± 0.15	(i) by substrate oxidation	0.40 ± 0.16
(ii) by proton leak	−0.34 ± 0.15	(ii) by proton leak	−0.26 ± 0.12
		(iii) by phosphorylation	−0.16 ± 0.06
		Control over P/O:	
		(i) by substrate oxidation	0.03 ± 0.18
		(ii) by proton leak	−0.68 ± 0.24
		(iii) by phosphorylation	0.61 ± 0.07
Elasticity to $\Delta\Psi$ of:		Elasticity to $\Delta\Psi$ of:	
(i) substrate oxidation	−2.48 ± 2.21	(i) substrate oxidation	−1.84 ± 1.81
(ii) proton leak	1.29 ± 0.76	(ii) proton leak	1.24 ± 0.73
		(iii) phosphorylation	1.18 ± 0.21
Fluxes (nmol O_2/min/g perfused tissue):		Fluxes (nmol O_2/min/g perfused tissue):	
(i) substrate oxidation	0.208 ± 0.015	(i) substrate oxidation	0.215 ± 0.023
(ii) proton leak	0.208 ± 0.015	(ii) proton leak	0.130 ± 0.020
		(iii) phosphorylation	0.085 ± 0.011

The fluxes and overall elasticities were calculated from plots of mitochondrial $\Delta\Psi$ versus mitochondrial oxygen consumption rate derived from experiments (see sample data, Fig. 2) on seven (for substrate oxidation system and proton leak elasticities) and three (for ATP turnover system elasticities) hindquarter preparations, as explained in the legend to Fig. 2 and in the text. The 'substrate oxidation', 'ATP turnover' and 'proton leak' subsystems refer to the three blocks of the simplified metabolic system depicted in Fig. 1. The control coefficients represent the means ± S.E.M. calculated as follows: the individual elasticities and fluxes from each of the seven experiments of type a (see Fig. 2 legend) were paired with each of the fluxes and elasticities determined from the three experiments of type b (see Fig. 2 legend). These were used to generate 7 × 3 sets of control coefficients – the means ± S.E.M. ($n = 3$) of which are shown in the Table – using the equations given by Brand et al. [8,19] as explained in the text. Note that the elasticities and fluxes shown in the table are the average fluxes and elasticities for each of the three subsystems. However, use of these fluxes and elasticities to calculate control coefficients gives essentially the same values as those shown in the table. State 4 refers to the conditions with excess oligomycin present.

3.5. The value of the mitochondrial protonmotive force (Δp) in the perfused hindquarter

The state 4 Δp of muscle mitochondria in the perfused hindquarter is around 155 ± 24 mV ($\Delta\Psi = 93 \pm 15$ mV; $\Delta pH = 62 \pm 19$ mV) [4], which is low compared to the value of around 170 mV obtained with isolated, resting rat liver cells [29]. The possible reasons for this are discussed in detail in Ref. [4], but appear not to include unphysiological substrate limitation of the perfused tissue due to omission of erythrocytes from the perfusion medium [4]. It is possible that substrate limitation of respiration and $\Delta\Psi$ may be a physiological mechanism in the intact tissue (see e.g. Refs. [30,31]) and that the 'low' Δp measured in our perfused hindquarter preparation is in fact a reflection of the situation in vivo. Thus the results presented in this paper appear to be physiologically relevant unless the reason for the low $\Delta\Psi$ value is that the TPMP only reaches a small (and non-representative) proportion of the respiring mitochondria in the perfused hindquarter.

4. Conclusion

We have determined the degree of control exerted by proton leak (and other processes) over resting respiration rate (and other variables). We conclude, in general, that the pattern of control in perfused muscle is similar to that already determined in isolated liver cells. Proton leak has significant control over resting oxygen consumption in perfused skeletal muscle as it has over the respiration rate of resting liver cells. Thus our work supports the hypothe-

sis that proton leak has significant control over SMR in rats. Since SMR is equivalent to rate of heat production, our data (together with that of others) indicate that mitochondrial proton leak may be an important thermoregulatory mechanism in mammals.

Acknowledgements

This work was supported by a research studentship (to D.F.S.R.) and a grant (to M.D.B.) from the Agriculture and Food Research Council.

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