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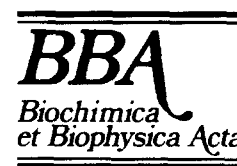

Control and kinetic analysis of ischemia-damaged heart mitochondria: which parts of the oxidative phosphorylation system are affected by ischemia?

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Abstract

We investigated the effects of ischemia on the kinetics and control of mitochondria isolated from normal and ischemic heart. The dependence of the respiratory chain, phosphorylation system and proton leak on the mitochondrial membrane potential were measured in mitochondria from hearts after 0, 30 min and 45 min of in vitro ischemia. Data showed that during the development of ischemia from the reversible (30 min) to the irreversible (45 min) phase, a progressive decrease in activity of the respiratory chain occurs. At the same time an increase in proton leak across the mitochondrial inner membrane was observed. Phosphorylation is inhibited but seems to be less affected by ischemia than respiratory chain or proton leak. Control coefficients of the 3 blocks of reactions over respiration rate were determined in different respiratory states between state 4 and state 3. Ischemia caused the control exerted by the proton leak to increase in state 3 and the intermediate state and caused the control by the phosphorylation system to decrease in the intermediate state. Taken together, these results indicate that the main effects of ischemia on mitochondrial respiration are an inhibition of the respiratory chain and an increase of the proton leak.

Keywords: Mitochondrion; Ischemia; Control coefficient; Heart

1. Introduction

Because of the central role of mitochondria in heart energy metabolism, numerous studies have addressed their pathophysiological role in the development of ischemic injury. Various events have been proposed to explain ischemic mitochondrial dysfunction such as losses of mitochondrial components (cytochromes *c* and *b* [1,2], etc.), decreased levels of mitochondrial adenine nucleotides [3,4], decreased activity of a variety of mitochondrial enzymes (ATP/ADP-translocator [5,6], ATPase [7], complex I [7–9], etc.). However, despite intensive study it is still unclear which of the effects of ischemia on different enzymes and processes are actually important in causing the impairment of the activity of oxidative phosphorylation — the main system providing energy for the myocardial cell. Usually the effects of ischemia on the individual components of oxidative phosphorylation are determined measuring maximal activities of these components, and thus it is unclear to what extent these changes in activity will affect the rate of the intact system.

One approach to investigate changes in kinetics of intact systems is the phenomenological kinetic analysis or elasticity analysis of Hafner et al. [10]. In this approach oxidative phosphorylation is conceptually divided into three subsystems: the mitochondrial respiratory chain, the phosphorylation system and the proton leak. The dependence of each of these three subsystems on their intermediate — the protonmotive force — is measured in the presence or absence of some physiological or pathological change in order to determine which of these subsystems is directly affected by the change. The same data can be used to

Abbreviations: TPMP, triphenylmethylphosphonium; $\Delta\Psi$, transmembrane difference in electric potential; $-z\Delta pH$, transmembrane difference in pH (in mV); Δp, protonmotive force; C_C, flux control coefficient of respiratory chain; C_P, flux control coefficient of phosphorylating system; C_L, flux control coefficient of proton leak across the mitochondrial inner membrane

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estimate the control coefficients of the subsystems over respiration using the top-down approach of metabolic control analysis [10]. We used these approaches to investigate the effects of ischemia on the kinetics and control of respiration in isolated heart mitochondria. The results show which subsystems of oxidative phosphorylation are affected by ischemia, and how important these effects are for changing the respiration rate.

2. Methods

2.1. Preparation of heart mitochondria

Ischemia was induced in vitro by autolysis as described in [11]: the hearts were washed from blood in warm (37°C) 0.9% KCl solution, placed in a humidified chamber and allowed to autolyse at 37°C in a thermostate for 30 min or 45 min. Mitochondria were isolated as described earlier [12] in a medium containing 180 mM KCl, 10 mM Tris, 1 mM EGTA, pH 7.7 (4°C). Mitochondrial protein was determined by the biuret method.

2.2. Measurement of oxygen consumption and mitochondrial membrane potential

Mitochondrial respiration rate and membrane potential were measured at 37°C in a 3 ml incubation chamber fitted with both a Clark-type oxygen electrode and a TPMP sensitive electrode. The incubation buffer contained 10 mM Tris-HCl, 5 mM KH_2PO_4, 1 mM EGTA, 20 mM dithiothreitol, 1 mM ATP, 10 mM succinate, 1 μM rotenone, 4 IU/ml creatine kinase, 1 mM free Mg^{2+} (calculated according to binding constants [13]), pH 7.2, 37°C. Different stationary rates of mitochondrial respiration were adjusted by varying creatine and creatine phosphate in the medium, and the concentration of KCl was changed accordingly for the maintenance of constant ionic strength [14]. Concentrations of creatine, creatine phosphate, KCl and $MgCl_2$ in the incubation buffer are given in the figure legends. Mitochondrial membrane potential was measured as described in [15] using a TPMP binding correction factor of 0.17 (μl/mg) for normal as well as for ischemia-damaged mitochondria. The binding correction factor was determined from the ratio of Rb^+ to TPMP accumulation as a function of mitochondrial volume (Fig. 1).

2.3. Determination of mitochondrial volume and pH gradient

Mitochondrial volume and pH gradient, $-z\Delta pH$, were determined from the distribution of ^3H_2O and [^{14}C]sucrose, and [^{3}H]acetate and [^{14}C]sucrose respectively as described previously [15] at several respiration rates from state 4 to state 3. Mitochondrial volumes were 0.64–0.70 μl/mg (normal), 0.63–0.68 μl/mg (30 min ischemia) and 0.75–0.78 μl/mg (45 min ischemia). ΔpH was found to be 12.4–19.1 mV (normal), 16.5–22.5 mV (30 min ischemia), 11.1–18.1 mV (45 min ischemia). Volumes and ΔpH did not significantly change in the media with different creatine/creatine phosphate ratios.

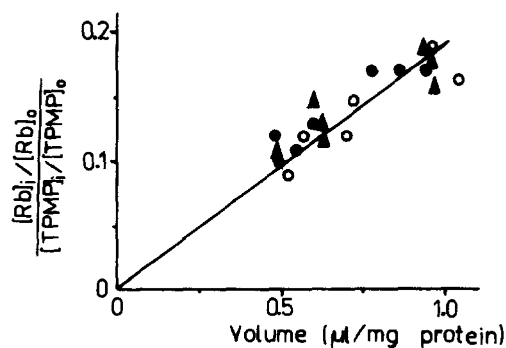

Fig. 1. Ratio of Rb^+ to TPMP accumulation as a function of mitochondrial volume. Mitochondria (1 mg protein) were incubated for 1 min at 37°C in 1 ml of medium containing various concentrations of sucrose (50–300 M) and 5 mM Hepes (pH 7.2, adjusted with LiOH), 5 mM succinate, 1 mM EGTA, 5 μM TPMP, 5 μM rotenone, 100 pmol/mg valinomycin, 1 μg/mg oligomycin, 0.1 μC_i /ml [^{3}H]TPMP, 0.04 μC_i /ml ^{86}Rb. Reactions were stopped by rapid centrifugation for 2 min and the supernatant and the pellet were assayed for radioactivity in a scintillation counter. ○, normal; ●, 30 min ischemia; ▲, 45 min ischemia. The slope of the line (0.17 μl/mg) is the TPMP binding correction.

2.4. Calculation of elasticities and control coefficients

The overall elasticities and control coefficients of the chain, phosphorylation and proton leak across inner mitochondrial membrane were calculated as described in [10] from the sensitivities of these fluxes to the membrane potential (see Fig. 3).

2.5. Statistical analysis

Data are expressed as means + S.E. of at least three experiments. Statistical comparison between control and ischemic data was performed using the unpaired Student's t-test. Statistical significance was assumed at $P < 0.05$.

3. Results

The system under investigation consists of isolated normal or ischemia-damaged rat heart mitochondria respiring in a steady state with succinate (Fig. 2). $\Delta\psi$ is generated by the respiratory chain, consisting of all the steps from externally added succinate to $\Delta\psi$. $\Delta\psi$ is consumed by the two branches of the system: the phosphorylation subsystem which includes all the steps from $\Delta\psi$ to creatine phosphate and the proton leak across mitochondrial inner membrane. The rates of respiration can be set at

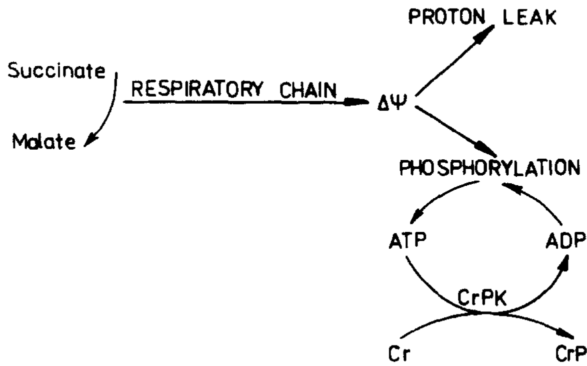

Fig. 2. Scheme of oxidative phosphorylation system in heart mitochondria. $\Delta\psi$ is produced by the respiratory chain (consisting of the dicarboxylate carrier, succinate dehydrogenase, CoQ and cytochromes). The reactions that consume $\Delta\psi$ are the phosphorylation subsystem (ATPase, ATP/ADP-translocator, phosphate carrier and, indirectly, the ADP-regenerating system) and the proton leak. Cr, creatine; CrP, creatine phosphate; CrPK, creatine phosphokinase.

different values by changing creatine and creatine phosphate in the incubation medium in the presence of excess of creatine phosphokinase. Δp is the true intermediate between these subsystems, but with phosphate in the medium it was found that ΔpH did not change significantly in any of the states investigated.

Fig. 3 shows an example of an experiment used to calculate the elasticities and control coefficients of the blocks of reactions of oxidative phosphorylation as described in Section 2. The broken line in Fig. 3 represents the kinetic response of the respiratory chain to the decrease

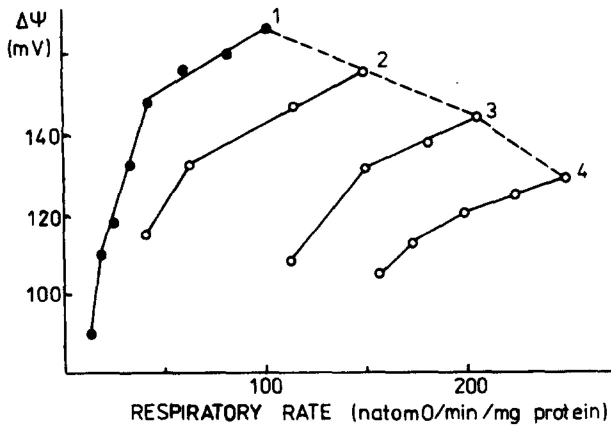

Fig. 3. Measurement of elasticities to membrane potential of $\Delta\psi$-producing and $\Delta\psi$-consuming reactions. Mitochondria were incubated in media (see Section 2) containing different ratios of creatine/creatine phosphate, KCl and total $MgCl_2$ (in mM), respectively: 1–5/45, 70, 2.64; 2–30/20, 90, 2.53; 3–40/10, 100, 2.33; 4–50/0, 110, 2.24. Five additions of TPMP were made to calibrate the TPMP-sensitive electrode (final TPMP concentration was 5 μM). Then 10 mM succinate was added. Respiration rate and $\Delta\psi$ were titrated by potassium malonate. Each point represents the mean of three determinations of respiration rate and $\Delta\psi$ on a single mitochondrial preparation. Data of one typical experiment on normal mitochondria are presented. ●, malonate titration of state 4 respiration in the presence of oligomycin; ○, malonate titration of phosphorylating respiration in media with different ratios of creatine/creatine phosphate.

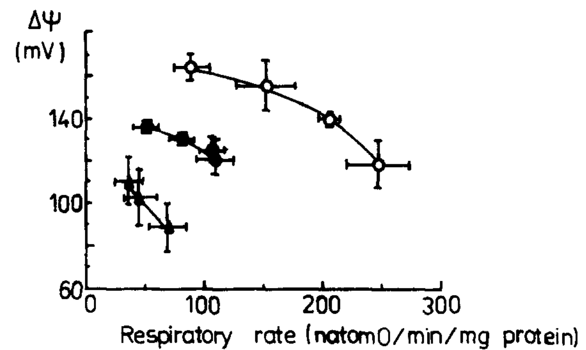

Fig. 4. Effect of ischemia on the kinetics of the respiratory chain. Mitochondrial respiration rate and $\Delta\psi$ were measured in the incubation media with different ratios of creatine/creatine phosphate (see Fig. 3). ○, normal; ●, 30 min ischemia; ▲, 45 min ischemia.

in the membrane potential induced by decrease of phosphorylation potential. This kinetic response can be used to investigate the direct effect of ischemia on the respiratory chain. Inhibition of the respiratory chain results in a decrease of the respiration rate at any given membrane potential or a decrease of membrane potential at any given respiration rate, as can be seen from the malonate titrations (solid lines in Fig. 3).

Fig. 4 shows the effect of ischemia on the kinetics of the respiratory chain. As can be seen from the figure, under the influence of ischemia the curve of dependence of mitochondrial respiration rate on membrane potential is shifted down and to the left. Longer periods of ischemia induces a more pronounced shift of the curve (compare curves for 30 min and 45 min ischemia). This means that during development of ischemia from the reversible (30 min) to the irreversible (45 min) phase a progressive decrease in activity of the respiratory chain occurs. At the same time, an increase in proton leak was observed (Fig. 5). Proton leak was determined as mitochondrial respiratory rate in state 4 in the presence of excess of oligomycin to inhibit phosphorylation. In this case, respiration rate is proportional to the rate of proton leak through the mito-

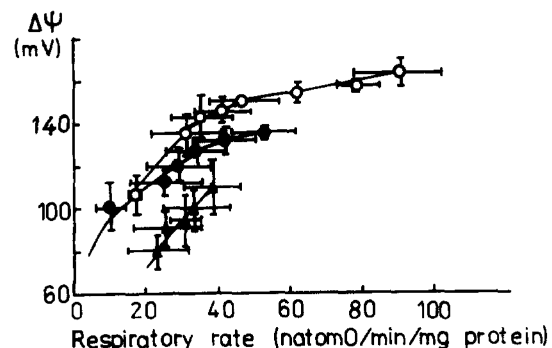

Fig. 5. Effect of ischemia on the kinetics of proton leak. State 4 respiration rate and $\Delta\psi$ were titrated by malonate in the presence of excess oligomycin (1 μg/mg protein) to prevent phosphorylation. ○, normal; ●, 30 min ischemia; ▲, 45 min ischemia.

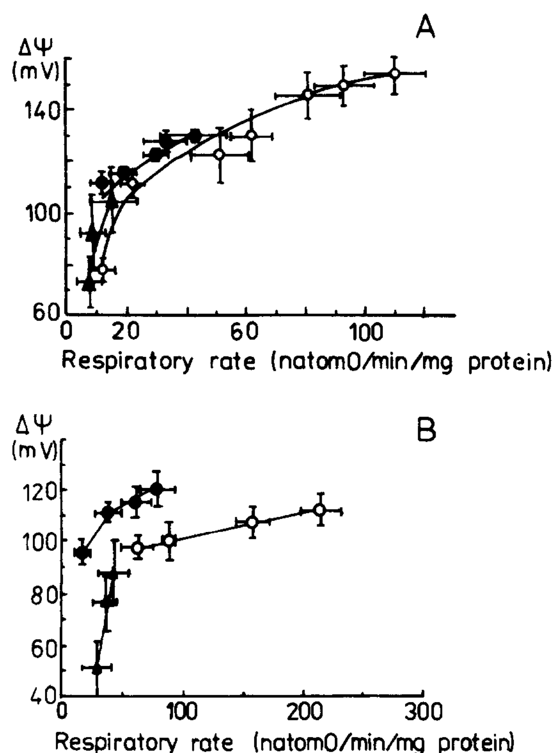

Fig. 6. Effect of ischemia on the kinetics of the phosphorylation system. (A) Malonate titration of $\Delta\psi$ and respiration rate in the intermediate state (creatine/creatine phosphate = 40 mM/10 mM). (B) Malonate titration of $\Delta\psi$ and state 3 respiration rate (creatine/creatine phosphate = 50/0 mM). ○, normal; ●, 30 min ischemia; ▲, 45 min ischemia.

chondrial inner membrane. As can be seen from Fig. 5 at each particular value of $\Delta\psi$ the respiration rate of ischemic mitochondria was higher than that of normal mitochondria indicating increased proton leak in ischemic mitochondria, particularly with 45 min ischemia.

Fig. 6 represents the kinetics of the phosphorylation subsystem of normal and ischemia-damaged mitochondria. The rate of phosphorylation was calculated at each particular value of $\Delta\psi$ as the difference between the respiration rate in the absence of oligomycin and in its presence. $\Delta\psi$ and respiration rate were changed by malonate titration. The data of Fig. 6A indicate that ischemia does not significantly change the kinetics of phosphorylation in the intermediate state between state 4 and state 3: the points for normal, 30 min and 45 min ischemia-damaged mitochondria almost lie on the same curve of dependence of respiratory rate on $\Delta\psi$. However, the activity of the phosphorylation subsystem in state 3 decreases after 30 min ischemia but not after 45 min (Fig. 6B). Thus, the activity of the phosphorylation subsystem seems to be less affected by ischemia than the respiratory chain or proton leak at least at intermediate states of respiration.

Data of Figs. 4–6 were used to calculate elasticities and control coefficients of the 3 blocks of reactions of oxidative phosphorylation over mitochondrial respiration rates at different states between state 4 and state 3 using equations described in [10].

Fig. 7 shows the control coefficients of the respiratory chain, proton leak and phosphorylation subsystem over the total rate of oxygen consumption by normal and ischemia-damaged mitochondria. In state 4 (Fig. 7A), the main control steps over oxidative phosphorylation in normal as well as in ischemia-damaged mitochondria are the respira-

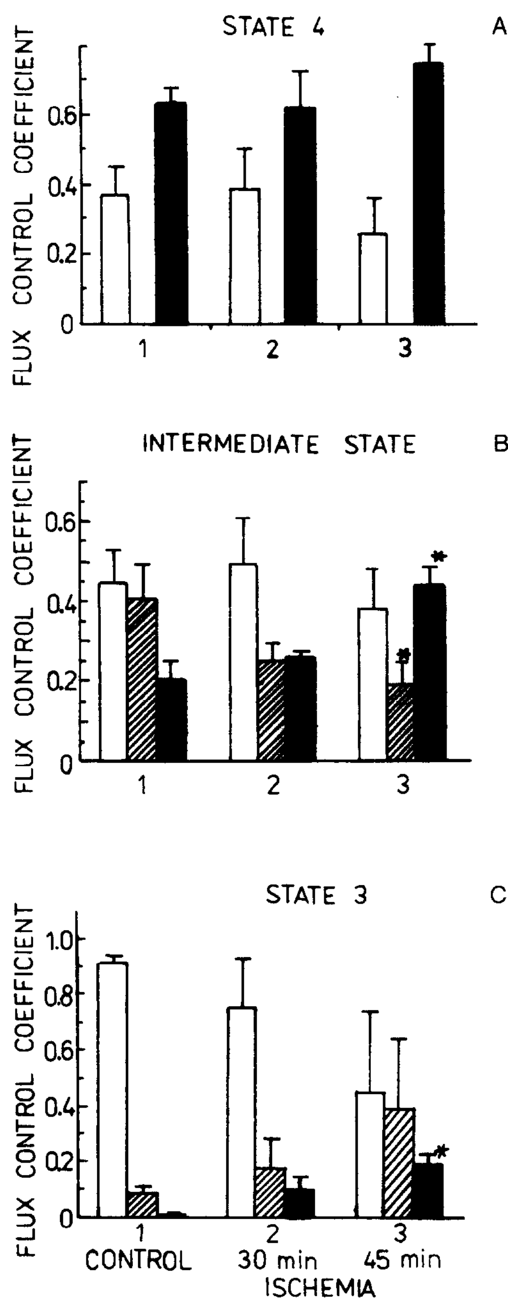

Fig. 7. Effect of ischemia on the distribution of control among blocks of reactions of oxidative phosphorylation in different states. Control coefficients over mitochondrial respiration rate exerted by the respiratory chain (□), the phosphorylation system (hatched bar) and proton leak (■) were calculated as described in Section 2.

tory chain and proton leak: $C_C = 0.37$, $C_L = 0.64$. The control coefficient of phosphorylation in state 4 is zero for normal and ischemic mitochondria. In the intermediate state (Fig. 7B) of normal mitochondria control is shared between the respiratory chain ($C_C = 0.45$), phosphorylation subsystem ($C_P = 0.41$) and, to a lesser extent, proton leak ($C_L = 0.19$). 45 min ischemia causes significant ($p < 0.05$) decrease of control coefficient of phosphorylation concomitantly with increase of control coefficient of the proton leak. A similar tendency was observed in the case of 30 min ischemia but changes were not statistically significant. In state 3 (Fig. 7C), the main control step of normal mitochondria is the respiratory chain ($C_C = 0.91$). The control by phosphorylation and proton leak is negligible ($C_P = 0.08$; $C_L = 0.02$). However, after 45 min ischemia the control coefficient of proton leak significantly increased ($C_L = 0.24$). The control coefficient of the phosphorylation subsystem apparently also increased and that of the respiratory chain decreased, but these changes were not statistically significant.

4. Discussion

We found that ischemia causes an inhibition of the respiratory chain, some inhibition of the phosphorylation system and an increase in proton leak. We also found that ischemia causes an increase in the control coefficient of the proton leak. This increase in control could be explained by the increased proton leak flux as a fraction of the total proton flux.

Previous measurements of the maximal activities of the individual components of oxidative phosphorylation have shown that ischemia causes: inhibition of complex I [8,9], complex II [1], complex III [1], ATPase [7], and the nucleotide translocator [5,6]. Ischemia also causes the loss of cytochrome *c* [1] and matrix adenine nucleotides [3,4]. It has been shown previously that ischemia causes an increase in the state 4 respiration rate [16] and an increase in the permeability of the inner mitochondrial membrane to protons (measured as mitochondrial swelling rate) [17]. But the functional significance of all these changes and their contribution to changing the respiration and ATP synthesis rates is not known.

Previous measurements of the control coefficient of the adenine nucleotide translocator over respiration rate in state 3 have shown that 45 min ischemia causes an increase in the control exerted by the translocator [18]. This may contribute to the apparent increase in control exerted by the phosphorylation system in state 3 seen in Fig. 7.

Our own data indicate that ischemia causes a large decrease in the activity of the respiratory chain, and that the respiratory chain has a high level of control over respiration rate in all states. Ischemia also causes an increase in the proton leak, but the proton leak only has significant control over respiration in state 4 and in the intermediate state. In some conditions ischemia causes an inhibition of the phosphorylation system, but this subsystem only has a high level of control over respiration rate in the intermediate state. Thus, we conclude that the inhibition of the respiratory chain is the major cause of the inhibition of respiration by ischemia, but the increase in proton leak and inhibition of the phosphorylation system may also contribute to the changes in respiration in particular conditions. However, it is important to note that this investigation used succinate as respiratory substrate and this excluded contribution of complex I and matrix enzymes.

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