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Response of heart mitochondria to hyperthermia: activation at the febrile temperature *versus* loss of the inner membrane barrier at higher temperatures

Rasa Žūkienė[1],

Zita Naučienė[1,2],

Jolita Čiapaitė[1],

Vida Mildažienė[1]*

[1] *Centre of Environmental Research, Faculty of Natural Sciences, Vytautas Magnus University, Kaunas, Lithuania*

[2] *Institute for Biomedical Research, Kaunas Medical University Kaunas, Lithuania*

Modular kinetic analysis was applied to compare the response of the oxidative phosphorylation system in rat heart mitochondria to different temperatures in a hyperthermic range (40–45 °C vs. 37 °C) aiming to identify the affected modules of the system. The results indicate that increasing the temperature in a febrile range (37–41 °C) slightly activated the mitochondrial function due to stimulation of the respiratory module, while the kinetics of the proton leak and the phosphorylation module was not affected. A very different pattern of changes was induced at slightly higher temperatures (42–45 °C). A severe impairment of the energy transforming function of mitochondria, caused by a sudden disturbance of the membrane barrier function, was observed at 42 °C. Although the state 3 respiration rate at this temperature remained unchanged, proton leak across the inner mitochondrial membrane was substantially increased, the respiratory module being slightly inhibited, and the membrane potential ($\Delta\psi$) decreased by 8 mV, leading to a partial uncoupling of oxidative phosphorylation and a diminished ATP synthesis (16% lower phosphorylation flux). The further increase of temperature was extremely harmful to the mitochondrial function: after three minutes of incubation at 45 °C, mitochondria became completely uncoupled and were unable to maintain $\Delta\psi$ and to synthesize ATP. To sum it up, energy production in heart mitochondria was slightly activated at temperatures in the febrile range and was drastically impaired at temperatures higher by only a few degrees.

Key words: heart mitochondria, modular kinetic analysis, membrane permeability, hyperthermia, oxidative phosphorylation

Abbreviations
TPP^+ – tetraphenylphosphonium ion; V_2 and V_3 – rates of mitochondrial respiration in state 2 and state 3, respectively; $\Delta\psi$ – transmembrane difference of electrical potential, J_O, J_P and J_L – respiration, phosphorylation and proton leak fluxes through the oxidative phosphorylation system.

INTRODUCTION

The events following exposure of cells to moderate heating involve an interplay of multiple factors operating at different regulatory levels and inducing changes in the metabolic activities, signal transduction, and gene expression. The death or survival of different cells upon hyperthermia is determined by the molecular mechanisms that are not yet well established. Despite its physiological importance, information on the molecular events induced by fever in various types of cells is scarce [1]. Suprahysiological hyperthermia (usually in the range 42–45 °C) is clinically applied in cancer treatment [2, 3], although quite little is known about differences in cellular response to heating under fever compared to more severe hyperthermic conditions. Multiple mechanisms may be responsible for the higher sensitivity of tumour cells to temperature treatment as compared to normal cells. Also, it is still not clear why cells of some tumours are more than other tumours sensitive to heating [4, 5], or why some normal types of cells (e. g., neurons, cardiomyocytes) are more sensitive that other normal cells [6–9].

It has been suggested that mitochondria are important players in the development of heat-stress-induced apoptosis in certain tumour cell lines [10, 11] as well as in cardiomyocytes [9]. These organelles perform multiple functions in cell life, including energy supply, maintaining ion balance, and up to transduction of apoptotic signals. However, the response of mitochondria from different tissues to heating was investigated only by a few research groups [9, 12–14]. Regardless of the mitochondrial origin, hyperthermia induces uncoupling of oxidative phosphorylation (increase in state 2 respiration) and a decrease in the efficiency of phosphorylation (drop in the ADP / O ratio), although

*Correspondence to: Vida Mildažienė, Prof. Dr. Habil., Centre of Environmental Research, Faculty of Natural Sciences, Vytautas Magnus University, Vileikos 8, LT-44404 Kaunas, Lithuania. E-mail: v.mildaziene@gmf.vdu.lt

the effect of hyperthermia on state 3 respiration to certain extent depends on mitochondrial origin.

However, the estimation of classical functional parameters (state 2 and 3 respiration or the ADP / O ratio) provides very limited information about the sites of action of possible multi-site effectors (such as hyperthermia) on the complex system of oxidative phosphorylation. Modular kinetic analysis (or top-down elasticity analysis) has been shown to be a valuable tool to identify the sites of action of multi-site effectors within complex systems (reviewed in [15]), including the effects of low temperatures on the system of oxidative phosphorylation [16]. Using this approach, a complex system (process) is simplified by division into a small number of modules involved in the production or consumption of one intermediate (so-called connecting or common intermediate). In our case, we divide oxidative phosphorylation into three modules (proton leak, respiratory and phosphorylation modules) that interact through the membrane potential $\Delta\psi$ as a connecting intermediate [17, 18]. In order to detect the components of the system that are influenced by the effector, the effector-induced shift in the kinetics of each module (i. e. the dependence of the flux through the module on the level of the connecting intermediate $\Delta\psi$) is determined.

The aim of our study was to perform modular kinetic analysis in the hyperthermic temperature range and to compare the effects on physiologically relevant fluxes of respiration, phosphorylation and proton leak induced by a febrile temperature (40 °C) and higher temperatures commonly used for hyperthermic treatment (42 and 45 °C). The results indicate that the response of heart mitochondria in these two ranges of elevated temperature is essentially different: the febrile temperature slightly activates mitochondrial energy producing functions, whereas only one degree above the fever temperature causes a severe impairment of the mitochondrial ability to the maintain membrane potential and to synthesize ATP.

MATERIALS AND METHODS

Isolation of mitochondria

Mitochondria were isolated from hearts of male Wistar rats (250–300 g) as described in [18], using an isolation medium containing 160 mM KCl, 20 mM Tris,10 mM NaCl, 5 mM EGTA, 1 mg/ml bovine serum albumine (pH 7.7). The animals were killed according to the rules defined by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Purposes (License No. 0006). After isolation, mitochondria were suspended in a buffer containing 180 mM KCl, 20 mM Tris, 3 mM EGTA (pH 7.3) and stored on ice. The concentration of the mitochondrial preparation was approximately 50 mg of mitochondrial protein per ml of stored suspension. Protein content was determined by the modified Biuret method.

Measurement of mitochondrial respiration and $\Delta\psi$

Mitochondrial respiration was measured at different temperatures (37, 40, 42 and 45 ± 0.1 °C) in a closed, stirred and thermostated 1.5 ml glass vessel equipped with a Clark-type oxygen electrode and a TPP^+ (tetraphenylphosphonium)-sensitive electrode (A. Zimkus, Vilnius University, Lithuania) allowing a simultaneous monitoring of $\Delta\psi$ and mitochondrial respiration. For each incubation, the TPP^+-sensitive electrode was calibrated with TTPBr to a final concentration of 267 nM. $\Delta\psi$ was calculated from the distribution of TPP^+ using the Nernst equation and a TPP+ binding correction factor of 0.16 µl/mg protein [18].

The assay medium contained 30 mM Tris, 5 mM KH_2PO_4, 110 mM KCl, 10 mM NaCl, 1 mM EGTA, 5 mM nitrilotriacetic acid, 1 mM dithiothreitol, 50 mM creatine, 5.17 mM $MgCl_2$ (1 mM free Mg^{2+}), and 0.875 mM $CaCl_2$ (1 µM free Ca^{2+}), pH 7.2. Excess of creatine kinase (0.1 mg/ml) was added to maintain steady state respiration. The experiments were performed using 1 mM pyruvate plus 1 mM malate as an oxidizable substrate. Mitochondria (0.3 mg protein/ml) were incubated in the assay medium with the respiratory substrate (state 2) for 3 min at 37, 40, 42 or 45 °C before state 3 respiration was initiated by addition of 1 mM ATP. In this study, the rate of mitochondrial respiration in state 2 (V_2), state 3 (V_3) and the respiratory control index (RCI = V_3/V_2) are defined according to the conventional terminology [19]. Mitochondrial swelling at different temperatures was determined spectrophotometrically [21] in the same medium.

Determination of dissolved molecular oxygen concentration

The concentration of molecular oxygen dissolved in the assay medium at different temperatures (37–47 ± 0.1 °C) was determined polarographically using a glucose oxidase catalyzed reaction between D-glucose and O2 [20] while the pH of the medium was strictly controlled at each temperature (pH 7.2). The reaction was initiated by adding 4.7 U/ml glucose oxidase solution and then 100 µM of glucose solution each minute until complete oxygen consumption. The concentration of dissolved oxygen was calculated from the obtained polarographic curves by the amount of glucose used for the reaction. The molar ratio coefficient of the reaction between D-glucose and O2 (1:0.823) was defined in the medium with a known concentration of dissolved oxygen at 37 °C [22].

Modular kinetic analysis

The mitochondrial oxidative phosphorylation system was conceptually divided into three functional modules interacting through the linking intermediate membrane potential ($\Delta\psi$) [17]: (i) the respiratory module that produces $\Delta\psi$ (comprised of the substrate carriers, dehydrogenases, respiratory chain complexes), (ii) the phosphorylation module that consumes $\Delta\psi$ (comprised of ATP synthase, adenine nucleotide translocator and phosphate carrier), and (iii) the proton leak module which also consumes $\Delta\psi$ (comprised of the passive permeability of the mitochondrial inner membrane to protons, and any cation cycling reactions).

The dependence of the flux through the respiratory module (J_O) on $\Delta\psi$ was determined by gradually inhibiting the flux through the phosphorylation module with the inhibitor of adenine nucleotide translocator carboxyatractyloside (0–1.75 nmol/mg protein) and in this way modulating the value of $\Delta\psi$, and concomitantly measuring the respiration flux J_O and $\Delta\psi$ corresponding to each new steady state. The dependence of the flux through the phosphorylation module (J_P) on $\Delta\psi$ was determined by titrating the flux through the respiratory module with the respiratory chain inhibitor rotenone (0–0.06 nmol/mg protein) and

concomitantly measuring the respiration flux (J_O) and Δψ corresponding to each new steady state. The phosphorylation flux J_P was calculated by subtracting the proton leak flux J_L from the respiration flux J_O at the same value of Δψ (i. e. $J_P = J_O - J_L$). The dependence of the flux through the proton leak module (J_L) on Δψ was determined by titrating the flux through the respiratory module with rotenone (0–0.08 nmol/mg protein) when the flux through the phosphorylation module was fully blocked by addition of excess oligomycin (0.2 mg/mg mitochondrial protein). The experiments of modular kinetic analysis were paired experiments: the measurements were performed at 37 and 40 °C, or at 37 and 42 °C using the same mitochondrial preparation.

Data presentation and statistical analysis

The results are presented as means ± SEM (n = 3). Statistical significance of the temperature effects was evaluated using Student's t test (paired). The differences were assumed to be statistically significant at $p < 0.05$.

RESULTS

Cardiac dysfunction and failure is the main cause of heat-related death [23], and it is accompanied by a substantial injury of cardiomyocytes, which is associated with changes in mitochondrial morphology and function followed by an impairment of cellular energy metabolism leading to a severe cell injury and death [9]. This implies the need to determine the kinetic response of mitochondria from healthy heart tissue to the febrile range as well as to the supra-physiological range of heating. We performed modular kinetic analysis (as in [17]) on isolated heart mitochondria with the aim to detect how the increase in temperature in the range from 37 to 45 °C affects the kinetics of the respiratory, phosphorylation and proton leak modules.

For the modular kinetic analysis to be correct, the system under investigation must be able to achieve a steady state [15]. For this reason, we evaluated the ability of isolated heart mitochondria to respire at a constant rate and to maintain Δψ in the course of their incubation in state 2 at an elevated temperature. We obtained (data not shown) that upon incubation for 7 min at 37 °C the rate of mitochondrial respiration in state 2 (V_2) and membrane potential Δψ was constant (73.5 ± 3.5 nmol O • min^{-1} • mg protein^{-1} and 164.1 ± 0.3 mV, respectively). These values were not significantly different at 40 °C (91 ± 7 nmol O • min^{-1} • mg protein^{-1} and 163.6 ± 0.3 mV, respectively). However, incubation at 42 °C (one degree above the upper limit of the fever temperature) induced a very fast increase in V_2 and a decrease in Δψ: after 5 minutes of incubation V_2 increased to 315 ± 24 nmol O • min^{-1} • mg protein^{-1}, and Δψ decreased to 130.4 ± 7.6 mV. This possibly indicates a progressively developing uncoupling due to the increasing permeability of the inner mitochondrial membrane. At 45 °C the uncoupling effect was immediate: after two minutes of incubation V_2 reached the maximal value of 414.5 ± 9.2 nmol O • min^{-1}• mg protein^{-1}. However, state 2 respiration was inhibited in the time course of the further incubation (after 5 min V_2 decreased to 335 ± 6 nmol O • min^{-1} • mg protein^{-1}). Meanwhile Δψ decreased to 97.9 ± 6.7 mV after three minutes of incubation. Thus, at 45 °C mitochondria are completely uncoupled and there is evidence for a time-dependent inhibition of the respiratory chain. It is worth noting that there was no mitochondrial swelling in the temperature range 37–45 °C.

From the obtained results we have concluded that the maximal temperature for applying modular kinetic analysis is 42 °C, since the further increase in temperature is related to a strong and time-dependent uncoupling. However, analysis at 42 °C is possible only with some reservations, i. e. if the rates and Δψ with different concentrations of inhibitors are compared in the same time points (e. g., standardized at 4th–5th minute) but in different runs.

The aim of the further experiments was to evaluate how temperature elevation from 37 °C to 40 and 42 °C affects the kinetic dependences of fluxes through the proton leak, the respiratory and the phosphorylation modules on Δψ in heart mitochondria oxidizing pyruvate plus malate. The results showed that increasing the temperature from 37 °C to 40 °C (Fig. 1, Table 1) had no effect on the kinetics of phosphorylation (Fig. 1, A) and proton leak (Fig. 1, C), but lead to a slight activation of the reactions of

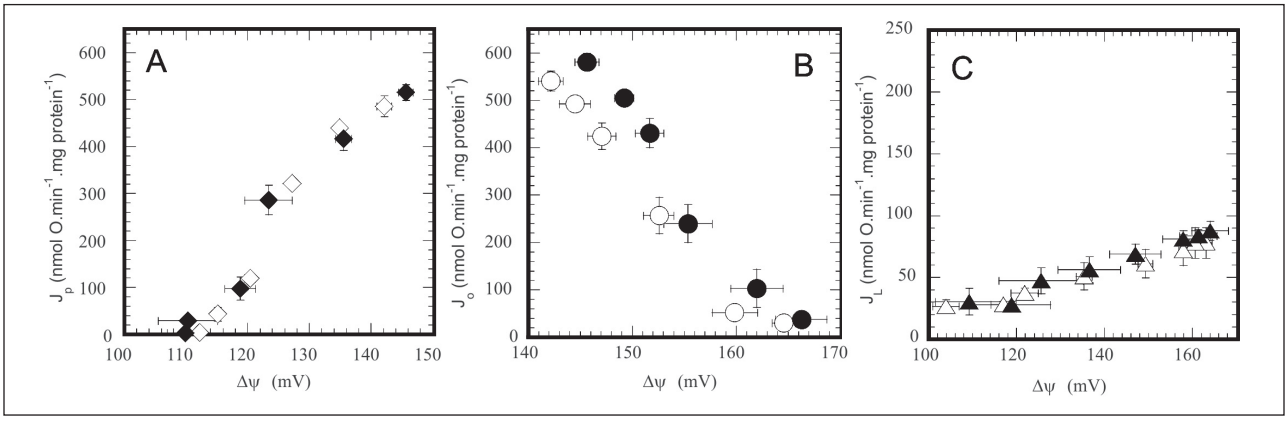

Fig. 1. Comparison of the kinetics of oxidative phosphorylation modules at 37 °C and 40 °C.

A – kinetics of phosphorylation module obtained by titration with rotenone. Phosphorylation flux J_p was calculated as $J_p = J_o - J_L$ at the same value of Δψ; B – kinetics of respiratory module obtained by titration with carboxyatractyloside; C – kinetics of proton leak module obtained by titration with rotenone when ADP phosphorylation was blocked with oligomycin. Averages from n = 3 independent experiments ± SEM. Open symbols, the experiments were carried out at 37 °C; closed symbols, the experiments were carried out at 40 °C

the respiratory module (Fig. 1, B). Although the respiration (J_O) and phosphorylation (J_P) fluxes through the modules of the system in state 3 did not change significantly, the increase in the Δψ value by 4 mV (Table 1) is consistent with the activation of the Δψ producing respiratory module. The increase in Δψ may be responsible for the higher value of the flux through the membrane leak module in state 3 (J_L). These results indicate the absence of uncoupling and a slight activation of mitochondrial oxidative phosphorylation by the febrile temperature.

When an analogous analysis was performed to compare the kinetics of oxidative phosphorylation modules at 37 and 42 °C, a surprisingly different pattern of changes was obtained (results shown in Fig. 2 and Table 2). The largest difference, in contrast to 40 °C (Fig. 1), is a strong activation of the flux through the proton leak module at 42 °C (Fig. 2, C). The flux through the proton leak module in state 3 (J_L) was increased more than 3-fold. In addition, the activity of the respiratory module was slightly suppressed (Fig. 2, B). As a result of the increased membrane leak and inhibition of the respiratory module, the value of Δψ in state 3 dropped by 8 mV (Table 2). It is important to note that although the kinetics of the phosphorylation module was not significantly affected (Fig. 2, A), the phosphorylation flux at the Δψ value corresponding to state 3 was reduced by 27%.

Table 1. **Effect of increasing the temperature from 37 °C to 40 °C on the fluxes through the modules of oxidative phosphorylation and the membrane potential. J_o, J_p, J_L, the fluxes of respiration, phosphorylation, and proton leak, respectively; Δψ, membrane potential. Averages from n = 3 independent experiments; ± SEM. * statistically significant difference, p < 0.05; ** statistically significant difference, p < 0.01.**

Parameters of oxidative phosphorylation	Temperature	
	37 °C	40 °C
J_o (nmol O • min^{-1} • mg protein^{-1})	540 ± 21	581 ± 18
J_p (nmol O • min^{-1} • mg protein^{-1})	486 ± 22	515 ± 17
J_L (nmol O • min^{-1} • mg protein^{-1})	55 ± 1	66 ± 1**
Δψ (mV)	142 ± 1	146 ± 1*

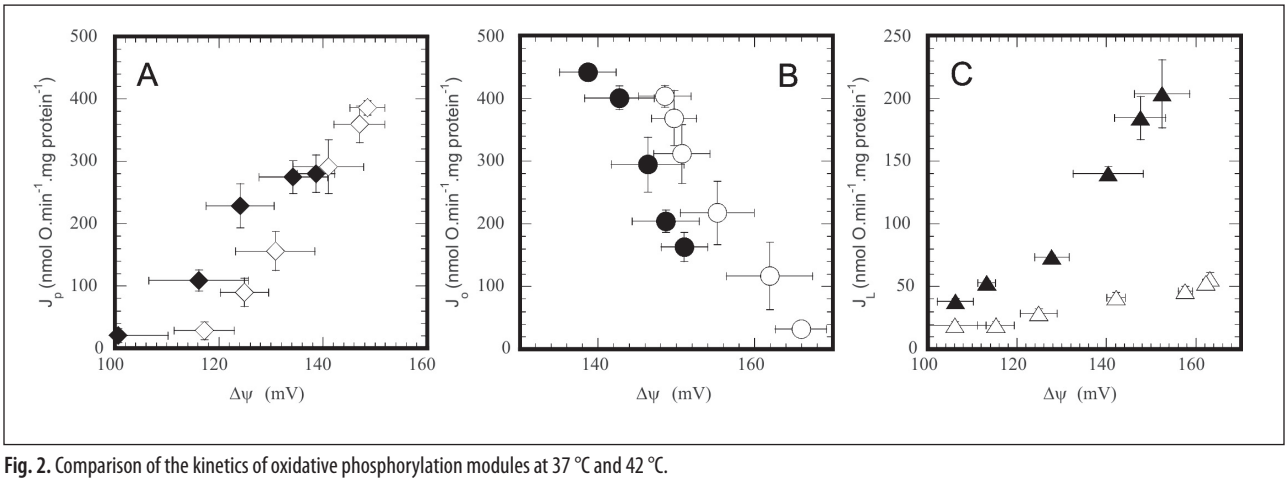

Fig. 2. Comparison of the kinetics of oxidative phosphorylation modules at 37 °C and 42 °C.
A – kinetics of phosphorylation module obtained by titration with rotenone. Phosphorylation flux J_p was calculated as $J_p = J_o - J_L$ at the same value of Δψ; B – kinetics of respiratory module obtained by titration with carboxyatractyloside; C – kinetics of proton leak module obtained by titration with rotenone when ADP phosphorylation was fully blocked with oligomycin. Averages from n = 3 independent experiments ± SEM. Open symbols, the experiments were carried out at 37 °C; closed symbols, the experiments were carried out at 42 °C

Table 2. **Effect of increasing the temperature from 37 °C to 42 °C on the fluxes through the modules of oxidative phosphorylation and the membrane potential. J_o, J_p, J_L, the fluxes of respiration, phosphorylation, and proton leak, respectively; Δψ, membrane potential. Averages from n = 3 independent experiments; ± SEM. *, statistically significant difference, p < 0.05; ** p < 0.01**

Parameters of oxidative phosphorylation	Temperature	
	37 °C	42 °C
J_o (nmol O • min^{-1} • mg protein^{-1})	404 ± 17	442 ± 12
J_p (nmol O • min^{-1} • mg protein^{-1})	386 ± 12	280 ± 30*
J_L (nmol O • min^{-1} • mg protein^{-1})	43 ± 2	140 ± 17**
Δψ (mV)	149 ± 3	139 ± 4*

DISCUSSION

Our study has demonstrated that elevation of temperature in the febrile range has a slight positive effect on the mitochondrial functions; however, with only one degree above the upper limit of that range, mitochondrial activity is severely challenged, mostly because of the progressive loss of the inner membrane barrier function. The increase in the inner membrane permeability, uncoupling and decrease in the ADP / O ratio was observed by all authors [9, 12–14] in the supra-physiological range of hyperthermia. However, there are some discrepancies in the effects observed in the febrile range since some authors have noted a significant uncoupling [9], whereas others have not [12–14]. Our results showed that the progressive increase in the membrane permeability to ions is not abolished by the inhibitor of the mitochondrial permeability transition pore cyclosporin A (data not shown). This indicates that the reasons other than opening of the permeability transition pore are responsible for the increase in the membrane leak and uncoupling of the oxidative phosphorylation in the supra-physiological range of temperatures.

Willis et al. [14] have reported that hyperthermia-induced uncoupling in isolated liver mitochondria is related to an abrupt change in the order of the inner membrane that occurs when the temperature rises above 42 °C. This could explain the differences in the effects of febrile and supra-physiological temperatures on the membrane permeability observed in our experiments.

The benefits of fever or an elevated body temperature (between 37 and 41 °C) for healing different morbid states, including infection by human immunodeficiency virus and cancer, are documented by numerous studies (reviewed in [2]). It has been shown that the evolutionary conserved response to infections improves the survival following infections because it enhances immune response [1] and decreases the viability of infectious agents [2]. Our results indicate that the mitochondrial function is slightly improved in the febrile range of temperatures, and this amelioration of cellular ATP supply to some extent may add to other beneficial effects of fever. The temperatures, even higher than febrile, are used in hyperthermic treatment protocols mostly for treating tumours; however, the mechanisms of temperature cytotoxicity and the reasons for the differences in response of normal and cancerous tissue are poorly understood. At a few degrees above febrile temperature, the mitochondrial inner membrane becomes permeable to ions, the membrane potential and the efficiency of oxidative phosphorylation decrease, therefore we conclude that at a supra-physiological temperature the mitochondrial functions are impaired and cellular energy supply severely declines. Although the rate of state 3 respiration seemingly remained the same (J_0 in Table 2), at 42 °C heart mitochondrial capability to phosphorylate was diminished due to uncoupling. However, this ability was completely lost when the temperature was raised from 42 to 45 °C. Thus, at higher temperatures the energy metabolism of a cardiac cell may be rapidly and severely challenged and threaten the survival of the cell.

It remains to be established to what extent mitochondrial response in other normal and tumour cells is different. Nevertheless, studies analogous to the one presented in this paper might provide the basis for a rational selection of regimens for hyperthermic treatment in different tissues. It is known that the benefitial apoptotic pathway of cell death is energy-dependent [24]. Therefore, it can be concluded from our results that milder treatment conditions (i. e. moderate hyperthermia) could be more beneficial since they lead to a less severe energy crisis and thus may be more preferable for inducing the apoptotic elimination pathway of tumour cells.

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Rasa Žūkienė, Zita Naučienė, Jolita Čiapaitė, Vida Mildažienė

ŠIRDIES MITOCHONDRIJŲ ATSAKAS Į HIPERTERMIJĄ: KARŠČIAVIMO TEMPERATŪRA AKTYVINA FUNKCIJAS, TAČIAU JĄ VIRŠIJUS VIDINĖ MEMBRANA NETENKA BARJERO SAVYBIŲ

S a n t r a u k a

Žiurkės širdies mitochondrijų oksidacinio fosforilinimo sistemos atsakas į temperatūros pokytį (37–45 °C) buvo įvertintas taikant modulių kinetikos analizę. Gauti rezultatai rodo, kad iki karščiavimo padidėjusi temperatūra (40 °C) dėl kvėpavimo modulio stimuliacijos truputį aktyvino mitochondrijų funkcijas, tačiau protonų laidumo ir fosforilinimo modulių kinetika nepakito. Visai kitokie rezultatai gauti temperatūrą padidinus dar keliais laipsniais (42–45°C). Esant 42°C temperatūrai, mitochondrijų energijos transformavimo funkcija stipriai sutrinka dėl staiga pablogėjusios membranos barjero funkcijos. Nors kvėpavimo greitis trečioje metabolinėje būsenoje nekinta, tačiau gerokai didėja mitochondrijų vidinės membranos laidumas protonams, slopinamas kvėpavimo modulis, 8 mV sumažėja membranos potencialas ($\Delta\psi$), dėl to substratų oksidacija iš dalies atskiriama nuo ADP fosforilinimo ir lėtėja ATP sintezė (fosforilinimo srautas sumažėja 16%). Toliau didinant temperatūrą mitochondrijų funkcijos tampa labai pažeidžiamos: po trijų minučių inkubacijos 45 °C temperatūroje substratų oksidacija būna visiškai atskirta nuo ADP fosforilinimo, mitochondrijos nesugeba palaikyti $\Delta\psi$ ir sintetinti ATP. Išvada: karščiavimo temperatūra skatina širdies mitochondrijų ATP sintezę, tačiau vos keliais laipsniais aukštesnė drastiškai sutrikdo mitochondrijų funkcijas.