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Hyperthermia modulates the effect of Ca^{2+} overload on respiration and NAD(P)H fluorescence in rat heart mitochondria

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We have compared the effect of moderate heating (42 and 45 ˚C) on the respiration and NAD(P)H fluorescence in isolated rat heart mitochondria incubated at two different Ca^{2+} concentrations (1 and 10 μM). The main effect of temperature elevation above the physiological level is an increased permeability of the inner mitochondrial membrane. This effect is pronounced at 42 ˚C and is followed by the secondary consequences – partial uncoupling, deenergizing, decrease in NAD(P)H and in the efficiency of oxidative phosphorylation. Although less effectively, mitochondria are capable to perform energy transformation at 42 ˚C. This ability is almost completely lost when the temperature is raised from 42 to 45 ˚C. At this temperature, mitochondria are almost completely uncoupled, they do not maintain the membrane potential and $NAD(P)H/NAD(P)^+$ equilibrium, hardly phosphorylate, and their respiratory chain is inhibited (the latter effect is more pronounced for glutamate + malate, but also evident for pyruvate + malate oxidation). Moderate heating significantly and progressively reduced the impairment of mitochondrial function by Ca^{2+} overload. In comparison to the high energy state at 37 ˚C, Ca^{2+} effect on respiration is much smaller in partially deenergized mitochondria at 42 ˚C and minimized at 45 ˚C when oxidative phosphorylation is almost completely uncoupled. Temperature effects are smoothened in Ca^{2+} overload (10 μM Ca^{2+}) inhibited mitochondria in comparison to mitochondria respiring at the optimal 1 μM Ca^{2+} concentration.

Key words: hyperthermia, Ca^{2+} overload, heart mitochondria, NAD(P)H fluorescence

INTRODUCTION

For many years hyperthermia has been known as a potential therapeutic option for the treatment of a variety of diseases, immunological and oncological included [1–2]. There are numerous evidences that hyperthermia can increase the effectiveness of other therapies [3–6]. The death or survival of different cells upon fever, extensive exercise or externally induced hyperthermia is determined by a multifactorial molecular mechanism which is not yet well defined. It has been shown that mitochondria are important in the development of heat-stress-induced apoptosis in certain tumour cell lines [7, 8]. It is supposed that these organelles belong to main ones injured by heat in cardiac myocytes [9]. Hyperthermia causes depolarisation of mitochondrial membrane and release of cytochrome c, and experimental evidences point to a possible importance of Ca^{2+} overload and permeability transition pore (PTP) in the hyperthermic response [8, 9]. The disbalance of Ca^{2+} homeostasis and the resulting mitochondrial Ca^{2+} overload are common for many pathological states (for review, see [10]). We have shown that accumulation of excessive Ca^{2+} leads to a very fast and rotenone-insensitive decrease in the mitochondrial $NAD(P)H/NAD(P)^+$ ratio [11]. This should promote the collapse of mitochondria, because the oxidized state of pyridine nucleotides strongly promotes PTP [12]. The opinions regarding the role of cytoplasmic Ca^{2+} ions in cellular response to heat stress are extremely controversial: some authors consider that the reported increase in the intracellular Ca^{2+} concentration plays a crucial role in the development of cell injury [13–16], whereas others conclude that changes in Ca^{2+} level during heating are not the principal factor in heat-induced cell death [17–

20]. In this work, we aimed to investigate how the response of heart mitochondria to hyperthermia is modulated by extramitochondrial Ca^{2+} concentration. We have compared the effect of moderate heating (42 and 45 ˚C) on the rate of respiration and NAD(P)H fluorescence in isolated rat heart mitochondria incubated at physiological (1 μM) and supra-physiological (10 μM) Ca^{2+} concentrations.

METHODS

Mitochondria were isolated from the heart of male Wistar rats and the protein in their suspension was estimated as described previously [11]. 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). Mitochondria were suspended in the buffer containing 180 mM KCl, 20 mM Tris, 3 mM EGTA (pH 7.3) and stored on ice.

Mitochondrial respiration was measured at different temperatures (37, 42 and 45 ˚C) in a closed, stirred and thermostated 1.0 ml vessel fitted with a Clark-type oxygen electrode in a medium containing 30 mM Tris, 5 mM KH_2PO_4, 110 mM KCl, 10 mM NaCl, 1 mM EGTA, 5 mM NTA, 50 mM creatine, mitochondria (0.5 mg protein) and/or 0.875 mM $CaCl_2$ (1 μM free Ca^{2+}) and 5.17 mM $MgCl_2$ (1 mM free Mg^{2+}) or 1.3 mM $CaCl_2$ (10 μM free Ca^{2+}), pH 7,2 as described in [11]. The [ATP]/[ADP] ratio was clamped by an excess of creatine kinase (0.1 mg/ml), 50 mM phosphocreatine. State 3 respiration was activated by addition of 1 mM ATP. The experiments were performed using 1 mM pyruvate + 1 mM malate or 5 mM glutamate + 5 mM malate as an oxidizable substrate. Mitochondria (0.5 mg protein/ml) were incubated in this medium with the respiratory substrate (state 2) for 3 min at 37, 42 or 45 ˚C before state 3 respiration was induced by addition of 1 mM ATP or the uncoupled state by adding 50 μM 2,4-dinitrophenol (DNP) in the presence of oligomycin (2 μg/mg protein) and 1 mM ATP. The rate of mitochondrial respiration in state 2 (V_2), state 3 (V_3) and the respiratory control index (RCI = V_3/V_2) were estimated according to the conventional technique [21]. For the measurement of mitochondrial membrane potential $\Delta\Psi$, the thermostated 1.5 ml polarographic vessel was fitted with a tetraphenylphosphonium (TPP^+) selective electrode (A. Zimkus, Vilnius University, Lithuania) using a 267 nM final concentration of TPP^+ in the medium. The membrane potential of mitochondria was calculated from the distribution of TPP^+ using a binding correction factor of 0.16 ml/mg [24]. NAD(P)H fluorescence was measured in the same vessel as was mitochondrial respiration. The experimental setup for NAD(P)H measurements consists of a Lumatec SUV-DC light source with a Computar lens (f = 25 mm, 1:1.8), a liquid wave guide and an intensified CCD camera CPL-22B (Canadian Photonics Labs). The excitation and emission wavelengths were 347 ± 4 nm and 467 ± 3 nm (after passing the band pass filters), respectively. Separate experiments using 10 μM NADH solution in 0.5% intralipid were performed to evaluate the temperature dependence of NAD(P)H fluorescence intensity. The obtained temperature correction factors 1, 1.09 and 1.1 were used for NAD(P)H fluorescence values at 37, 42 and 45 ˚C, respectively.

Dissolved molecular oxygen at different temperatures (32, 37, 42 and 47 ˚C) was determined using glucose oxidase catalyzed reaction between D-glucose and O_2 [22] in the incubation medium described above, while the pH of the medium was strictly controlled at each temperature (pH 7.2). Polarographic curves were obtained by adding 4.7 U/ml glucose oxidase solution, and then 10 μM 10 ml glucose solution was added every minute until complete oxygen consumption. The concentration of dissolved oxygen was calculated from the amount of glucose used for the reaction.

The results are presented as means ± S.E. (n = 6). Statistical analysis was performed using Student's test, and $p < 0.05$ was taken as the level of confidence.

RESULTS

Cardiac dysfunction is supposed to be the main cause of heat-related death [23]. Substantial injury of cardiomyocytes by heat treatment is associated with destructive changes in mitochondrial morphology and function, which are followed by impairment of cellular energy metabolism leading to severe cell injury and death [9]. Experimental evidences for Ca^{2+} overload, oxidative stress and facilitated PTP opening in cardiac mitochondria isolated from heated cardiomyocytes were obtained [9]. However, the question of the possible contribution of changes in cytoplasmic Ca^{2+} concentration to hyperthermic injury of cell remains to be a matter of debate [13–20]. We aimed to investigate how the response of mitochondria to heat treatment is modulated by the extramitochondrial concentration of Ca^{2+} ions. To this end, we determined NAD(P)H fluorescence and the rate of respiration in different metabolic states (state 2, state 3 and the uncoupled state) in isolated rat heart mitochondria oxidizing two substrates – pyruvate + malate and glutamate + malate at 37, 42 and 45 ˚C in a medium containing different Ca^{2+} concentrations. The results of the preliminary experiments indicated that the response of mitochondrial respiration to temperature did not depend on extramitochondrial Ca^{2+} concentration when it was changed from 5 nM to 1 μM (data not shown). Therefore we compared temperature effects on mitochondrial functions at 1 μM and 10 μM Ca^{2+} concentrations in the medium. The first Ca^{2+} concentration may be considered as physiological or optimal for stimulation of respiration with most substrates [24], whereas the second concentration is supra-physiological, related to Ca^{2+} overload and a pronounced inhibition of mitochondrial respiration [25].

The results (Fig. 1) indicate that the effect of temperature on oxygen consumption in mitochondria depends on the metabolic state of mitochondria and, to some

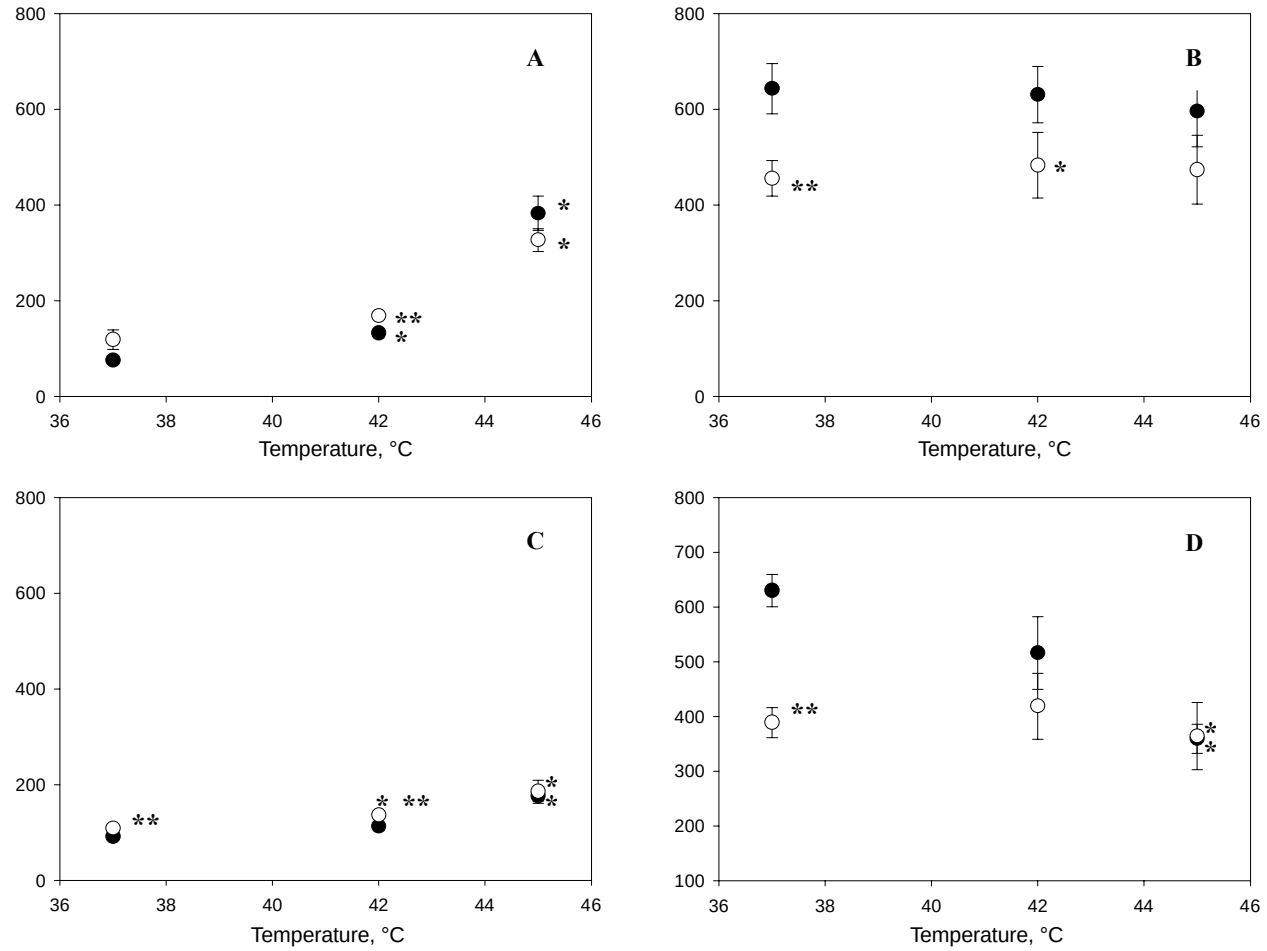

Fig. 1. The dependence of the respiration rate in heart mitochondria on temperature
A – state 2 respiration with pyruvate + malate (n = 6); B – state 3 respiration with pyruvate + malate (n = 6); C – state 2 respiration with glutamate + malate (n = 6); D – state 3 respiration with glutamate + malate (n = 6); ● – 1 μM Ca^{2+}, ○ – 10 μM Ca^{2+}; * – statistically significant temperature effect, ** – statistically significant Ca^{2+} effect.

extent on the substrate for respiration. The increase of temperature above the physiological value (from 37 to 42 and 45 ˚C) significantly increased state 2 respiration both at 1 μM and 10 μM Ca^{2+}, indicating a strong uncoupling. This effect was more pronounced at a higher temperature range – an increase of temperature by 5 degrees (from 37 to 42 ˚C) induced a rise in state 2 respiration rate by 74% at 1 μM Ca^{2+}, whereas the further elevation of temperature by 3 degrees (from 42 to 45 ˚C) resulted in almost three times higher respiration with pyruvate + malate. The corresponding numbers for glutamate + malate were 23% (from 37 to 42 ˚C) and 57% (from 42 to 45 ˚C) activation of state 2 respiration. Thus, the respiration at 45 ˚C was not several-fold higher than at 37 ˚C, as it was for pyruvate + malate (Fig.1A), i. e., temperature affected state 2 respiration with glutamate + malate much less than with pyruvate + malate. This difference implies that uncoupling is not the only temperature effect on the oxidation of different substrates.

To elucidate the possible effects on the respiratory chain in the mitochondria oxidizing pyruvate + malate and glutamate + malate, we determined temperature-induced changes in uncoupled respiration with both substrates. The results showed that the rise of temperature from 37 to 42 ˚C did not affect the rate of pyruvate + malate oxidation in mitochondria uncoupled by DNP – it was 612 ± 49 and 618 ± 61 (1 μM Ca^{2+}) and 397 ± 22 and 414 ± 50 nmol O/min per mg protein (10 μM Ca^{2+}) at 37 and 42 ˚C, respectively. Uncoupled respiration with glutamate + malate was increased with the same rise in temperature from 582 ± 50 to 716 ± 40 (1 μM Ca^{2+}) and from 387 ± 44 to 462 ± 9 nmol O/min per mg protein (10 μM Ca^{2+}). However, uncoupled respiration with both substrates substantially decreased at 45 ˚C: the rate with pyruvate + malate was only 390 ± 22 (1 μM Ca^{2+}) and 341 ± 30 nmol O/min per mg protein (10 μM Ca^{2+}), with glutamate + malate – 291 ± 27 (1 μM Ca^{2+}) and 292 ± 21 (10 μM Ca^{2+}). Thus, the inhibitory effect of 45 ˚C was more pronounced at a low concentration of Ca^{2+} and stronger for glutamate + malate (-50 and -19%) was than for pyruvate + malate oxidation (-36 and -6%, at 1 and 10 μM Ca^{2+}, respectively). The inhibitory effect of Ca^{2+} overload on the uncoupled rate was also diminished at 45 ˚C: for pyruvate + malate it was -35, -33 and -5%, for glutamate + malate -34, -35%, and zero at 37, 42 and 45 ˚C, respectively.

We have shown earlier that Ca^{2+} overload at 37 ˚C partially uncouples mitochondrial respiration [25] and that is explained by activation of Ca^{2+} cycling. In line with previous observations, our results show that at 37 ˚C the increase in Ca^{2+} concentration from 1 to 10 μM is related to a higher rate of respiration with both substrates in state 2 (Fig. 1A and C). However, the ability of Ca^{2+} to uncouple mitochondria decreases with temperature: the effect of Ca^{2+} ions on oxygen consumption in state 2 is +57, +28, -15% (pyruvate + malate) or +20, +21 and +5% (glutamate + malate) at 37, 42 and 45 ˚C, respectively. We explain this by a strong dependence of Ca^{2+} uptake to mitochondria on the membrane potential [26] which is dissipated due to uncoupling pronounced at 42 ˚C and very strong at 45 ˚C. The measurements of the membrane potential of mitochondria respiring with pyruvate + malate (performed after three minutes of incubation at a certain temperature) confirm this suggestion. The increase of temperature from 37 ˚C to 42 ˚C decreased $\Delta\Psi$ in state 2 by 12 mV (from 156 ± 2 to 144 ± 1 mV), and in state 3 – by 21 mV (from 136 ± 2 to 115 ± 7 mV) in the medium containing 1 μM Ca^{2+}. The membrane potential at 10 μM Ca^{2+} was lower than at 1 μM Ca^{2+}, and the same rise in temperature induced a bit smaller reduction of membrane potential both in state 2 (from 136 ± 3 to 128 ± 2 mV) and in state 3 (from 126 ± 5, 108 ± 4 mV). A continuous loss of $\Delta\Psi$ in mitochondria was observed during their incubation at 45 ˚C, so that in three minutes the membrane potential dropped from the initial value 137 ± 1 to 74 ± 7 mV (in state 2, at 1 μM Ca^{2+}) or from 136 ± 1 to 66 ± 5 mV (in state 2, at 10 μM Ca^{2+}) and the further transition to from state 2 to state 3 was followed by a very negligible $\Delta\Psi$ decrease. It is important to note that both at 1 μM and 10 μM Ca^{2+} mitochondria are still capable to phosphorylate at 42 ˚C (transition from state 2 to state 3 is followed by clear drop in $\Delta\Psi$). However, our results show that this transition is severely impaired at 45 ˚C, possibly because strong uncoupling lowers the membrane potential to the values hardly compatible with the operation of the phosphorylation machinery.

One may note that the increase in Ca^{2+} concentration from 1 to 10 μM did not change the pattern of temperature effects on respiration in state 2 with glutamate + malate (Fig. 1C) and slightly smoothened the effect of temperature with pyruvate + malate (Fig. 1A). However, increase in Ca^{2+} concentration had an evident inhibitory effect on state 3 respiration with both substrates (Fig. 1B and D). This finding is in line with our previous results [25] indicating that Ca^{2+} overload strongly inhibits the oxidation of NAD-dependent substrates at 37 ˚C in heart mitochondria. The rate of respiration in state 3 at 37 ˚C with pyruvate + malate was by 29% and with glutamate + malate by 40% lower at 10 μM as compared to the rate at 1 μM Ca^{2+}. Increase in temperature reduced the inhibitory Ca^{2+} effect on state 3 respiration, so that it was statistically not significant (with pyruvate + malate -20 and -15%; with glutamate + malate -21% and zero at 42 and 45 ˚C, respectively). On the other hand, one may notice that an increase in temperature seemingly has no effect on state 3 respiration with pyruvate + malate (Fig 1B). However, state 3 respiration with glutamate + malate (Fig. 1D) at 1 μM Ca^{2+} is significantly (by 43%) inhibited with rise in temperature to 45 ˚C.

We explain temperature effects on state 3 respiration by several reasons. With the rise of temperature, due to the progressively increasing uncoupling of mitochondria (Fig. 1A and C) the membrane potential is lowered and the phosphorylation rate respectively decreases. The value of the respiratory control index RCI [21] for pyruvate + malate at 1 μM Ca^{2+} drops from 8.48 ± 0.64 (37 ˚C) to 4.8 ± 0.3 (42 ˚C) or 1.54 ± 0.06 (45 ˚C); at 10 μM Ca^{2+} it drops from 4.2 ± 0.7 (37 ˚C) to 2.9 ± 0.5 (42 ˚C) or 1.4 ± 0.1 (45 ˚C), whereas for glutamate + malate at 1 μM Ca^{2+} – from 6.96 ± 0.26 (37 ˚C) to 5.00 ± 0.26 (42 ˚C) or 2.00 ± 0.06 (45 ˚C); at 10 μM Ca^{2+} – from 3.42 ± 0.31 (37 ˚C) to 3.0 ± 0.4 (42 ˚C) or 1.83 ± 0.15 (45 ˚C). These results invariably show a severe loss of the efficiency of oxidative phosphorylation at higher temperatures, which is attributable to an increased relative contribution of the membrane leak to state 3 respiration and corresponding diminution of the "phosphorylating respiration". For pyruvate + malate the decrease of the latter is compensated by the increase in the uncoupled respiration rate, so that the overall rate of oxygen consumption in state 3 seems to be not affected by the temperature (Fig. 1B). Temperature effect on glutamate + malate oxidation in state 3 is stronger and, besides uncoupling, we explain this by a very additional inhibitory effect on the respiratory chain at 45 ˚C (as evidenced by the results obtained in the uncoupled state, in states 2 and 3, compare Fig. 1A to 1C, and 1B to 1D). A very similar pattern of temperature effect on glutamate oxidation was obtained for liver mitochondria (data not shown).

We have compared the effect of moderate heating on NAD(P)H fluorescence in isolated rat heart mitochondria incubated at various Ca^{2+} concentrations. The results (Fig 2, A and B) show that at the physiological temperature (37 ˚C) Ca^{2+} overload significantly reduces the NAD(P)H fluorescence, but the induction of state 3 respiration by ATP addition is followed by a clear shift towards a more oxidized state of mitochondrial pyridine nucleotides both at 1 μM and 10 μM Ca^{2+}. The values of NAD(P)H fluorescence progressively decreased with the rise of temperature, and this is explainable by the temperature-induced uncoupling. The initial NAD(P)H fluorescence at two Ca^{2+} concentrations was very similar at 42 ˚C, however, the course of the following changes was different – while the level of NAD(P)H reduction was stable during 3 min of incubation at 1 μM Ca^{2+} (middle curve in Fig. 2A), a continuous and fast NAD(P)H decrease occurred at 10 μM Ca^{2+} (Fig. 1B), so that the ATP addition induced NAD(P)H drop only at 1 μM Ca^{2+}. The increase in NAD(P)H fluorescence at the 6^{th} min of incubation at 1 μM Ca^{2+} is possibly caused by the complete consumption of

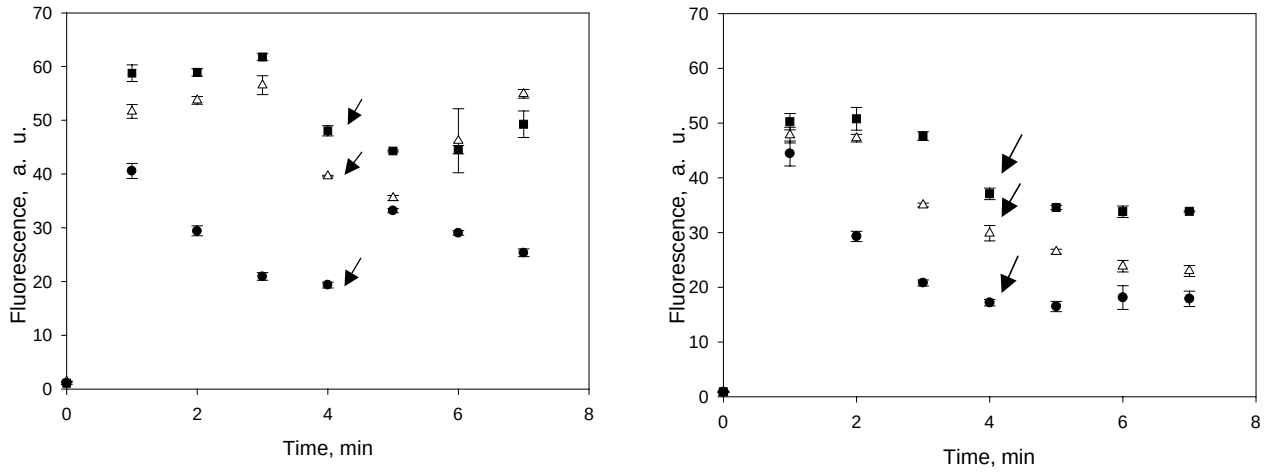

Fig. 2. The kinetics of the NAD(P)H fluorescence changes in heart mitochondria oxidizing pyruvate + malate during polarographic run at various temperature

A – mitochondria incubated in the medium containing 1 μM Ca^{2+}; B – the same at 10 μM Ca^{2+}. ■, △, ● – incubation at 37, 42, and 45 ˚C, respectively. Arrows show the moment of transition from state 2 to state 3 induced by ATP addition (see "Methods"). The averaged results of typical experiment are shown; similar results were obtained in three other experiments.

oxygen, followed by a reduction of the mitochondrial NAD^+ pool. A fast de-crease of NAD(P)H fluorescence at 45 ˚C was observed at both Ca^{2+} concentrations, possibly reflecting a continuous loss in the membrane potential at this temperature. Our results show that changes in NAD(P)H fluorescence are a sensitive and convenient marker of mitochondrial response to hyperthermic treatment.

DISCUSSION

The elucidation of the molecular mechanism of cell response to moderate heating is of importance for understanding the events that occur in the cell upon the use of heating for therapeutic purposes or during illnesses associated with fever. Our data indicate that the first manifestation of the effect of temperature elevation above the physiological level in mitochondria is an increase in the membrane permeability, which is pronounced already at 42 ˚C. This finding is in principal agreement with the results of other studies performed on isolated mitochondria [27, 28] or cardiomyocytes [9]. Partial uncoupling, deenergizing, a decrease in NAD(P)H and in the efficiency of oxidative phosphorylation are important secondary consequences of this effect. Although the function of mitochondrial is less effective, they can still perform the energy transduction function at this temperature. The picture changes drastically when the temperature is raised further from 42 to 45 ˚C. At this temperature mitochondria are almost completely uncoupled, they do not maintain the membrane potential and NAD(P)H/$NAD(P)^+$ equilibrium, are hardly able to phosphorylate and their respiratory chain is inhibited (the latter effect is more pronounced for glutamate + malate, but is also evident for pyruvate + malate oxidation).

The results concerning the dependence of the response of heart mitochondria to hyperthermia on extramitochondrial Ca^{2+} concentration are rather unexpected. We show that temperature effects are more pronounced when mitochondria respire at an optimal Ca^{2+}-concentration. Temperature effects seem smoothened in Ca^{2+} overload inhibited mitochondria. In this study, we demonstrate that moderate heating has significantly and progressively reduced mitochondrial injury by Ca^{2+} overload. We explain this by a decrease in mitochondrial ability to accumulate Ca^{2+} ions via electrogenic Ca^{2+} uniporter due to the increased membrane permeability and lowered membrane potential. Therefore, in comparison to the high energy state at 37 ˚C, Ca^{2+} effects on respiration are much smaller in partially deenergized mitochondria at 42 ˚C and negligible at 45 ˚C when oxidative phosphorylation is almost completely uncoupled.

It remains to be determined whether in the cellular environment the mode of temperature dependence and the pattern of induced dysfunction are the same as in isolated mitochondria. An important note is a striking increase in the severity of impairment of mitochondrial function with the rise of temperature by only a few degrees above 42 ˚C. The molecular reason for this effects is most possibly related to the drastic changes in the physical state of the membrane, but it remains to be elucidated. The observed pattern of temperature effects on mitochondrial function may provide useful information for the development of protocols for hyperthermic treatment of cancer. The central goal of this treatment is destruction of tumour cells along with protection of the neighboring healthy tissue. Bearing in mind that the apoptotic death pathway is energy-dependent, our results imply that a longer heating at a lower temperature might be more advantageous than a short but stronger heating leading to a less beneficial necrotic outcome due to the lack of energy caused by the complete default of mitochondrial ATP synthesis.

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HIPERTERMIJA KEIČIA CA^{2+} PERKROVOS POVEIKĮ ŠIRDIES MITOCHONDRIJŲ KVĖPAVIMUI IR NAD(P)H FLUORESCENCIJAI

S a n t r a u k a

Palyginome nuosaikaus kaitinimo (42 ir 45 ˚C) poveikį izoliuotų širdies mitochondrijų kvėpavimui ir NAD(P)H fluorescencijai inkubacijos terpėje esant dviem skirtingoms Ca^{2+} jonų koncentracijoms (1 ir 10 μM). Mūsų duomenimis, didinant temperatūrą virš fiziologinės reišmės (37 ˚C), pirmiausia stebimas vidinės mitochondrijų membranos pralaidumo didėjimas. Šis poveikis yra ženklus jau 42 ˚C temperatūroje ir lemia svarbius antrinius padarinius – dalinį atskyrimą, mitochondrijų deenergizavimą, NAD(P)H ir oksidacinio fosforilinimo efektyvumo mažėjimą. Nors ir mažiau efektyviai mitochondrijos geba transformuoti energiją esant 42 ˚C, tačiau jos beveik neatlieka šios funkcijos, kai temperatūra padidinama nuo 42 iki 45 ˚C. Esant šiai temperatūrai, oksidacinis fosforilinimas beveik visai atskirtas, mitochondrijos neišlaiko membraninio potencialo ir $NAD(P)H/NAD(P)^+$ pusiausvyros, menkas fosforilinimo aktyvumas, slopinama kvėpavimo grandinė (pastarasis poveikis ženklesnis mitochondrijoms oksiduojant glutamatą + malatą, tačiau būdingas ir piruvato + malato oksidacijai). Nuosaikus kaitinimas progresuojančiai mažino slopinantį Ca^{2+} perkrovos poveikį mitochondrijoms. Lyginant su energizuota mitochondrijų būsena, kai temperatūra siekė 37 ˚C, Ca^{2+} poveikis kvėpavimui buvo daug mažesnis deenergizavus mitochondrijas 42 ˚C temperatūroje. Šis poveikis sumažėjo iki minimalaus 45 °C temperatūroje, kai mitochondrijų oksidacinis fosforilinimas beveik visai atskirtas. Temperatūros didinimo poveikis Ca^{2+} perkrovos (10 μM Ca^{2+}) nuslopintoms mitochondrijoms taip pat buvo mažesnis nei kvėpuojančioms esant optimaliai 1 μM Ca^{2+} jonų koncentracijai.