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Mitochondrial Membrane Barrier Function as a Target of Hyperthermia

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Key words: *mitochondria; membrane permeability; hyperthermia; hyperthermic perfusion; modular kinetic analysis.*

Summary. *Background and Objective. Hyperthermia is a promising modality for cancer treatment that urgently requires detailed knowledge on molecular and cellular processes for the rational development of treatment protocols. The thorough study of the response of the inner membrane of heart and liver mitochondria to hyperthermia was performed in order to establish the pattern of the hyperthermia-induced changes in the membrane barrier function.*

Material and Methods. The isolated mitochondria from rat heart and liver (of both genders) were used for experiments, as well as mitochondria isolated from the perfused male rat liver. Changes in the membrane permeability were evaluated by mitochondrial respiration in state 2 or by estimation of the modular kinetics of the membrane leak.

Results. The inner membrane of isolated mitochondria from healthy tissues was found to be an extremely sensitive target of hyperthermia that exerted the response even in the febrile range. More severe hyperthermia compromised the inner mitochondrial membrane function; however, this response was tissue-specific and, to some extent, gender-dependent (for liver mitochondria). The data obtained by direct heating of isolated mitochondria were validated by experiments on the perfused liver.

Conclusions. The obtained results imply a crucial importance of the evaluation of the tissue- and gender-specific differences while developing or improving the protocols for hyperthermic treatment or combinatory therapy.

Introduction

Although hyperthermia may be considered as a separate modality for cancer treatment, it is especially recognized for its synergistic action with the conventional cancer therapies such as radiation and chemotherapy. Combination with hyperthermia improves the results of cancer treatment and decreases the side effects of conventional therapies (1–3). Therefore, the protocols for hyperthermic treatment are under a continuous development that requires basement on the knowledge of molecular and cellular processes. It is important to establish thresholds for thermal damage in human tissues that vary among tissue species as well as among healthy and diseased tissues (4). Induced thermal damages in the healthy organs set certain limits for the application of hyperthermia, in particular, for the whole body hyperthermia. Mild hyperthermic conditions in the healthy liver tissue are also created around the central coagulative zone during thermoablation of hepatic tumors (5–7) and during catheter radiofrequency ablation in the heart, which is considered a standard therapy for a wide variety of cardiac arrhythmias (8).

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The death or survival of different cells on heating is determined by multifactorial molecular mechanisms that strongly depend on the applied temperature: signal transduction pathways are affected, numerous changes in the structure and function of cellular membranes, proteins, and organelles are induced, and protein expression changes rapidly in response to stress (9). Comparison of the thermal susceptibility of various biomolecules has revealed that the plasma membrane is the most sensitive to heating among all cellular components (10) – thermal alteration of the plasma membrane is the earliest event in cellular response to hyperthermia and may be considered as the most significant cause of the following cell injury. The response of other cellular membranes has not been more thoroughly studied in this respect yet.

Mitochondria are highly dynamic organelles that exhibit morphological as well as biochemical changes during physiological cell metabolism and stress responses (11, 12). These organelles may capture a central directory in the cell fate when affected by physical, biochemical, or environmental stress factors. However, the response of mitochondria from different normal tissues to heating has been investigated only by few research groups. It was shown that regardless of mitochondrial origin, hyperther-

mia induced the uncoupling of oxidative phosphorylation (an increase in state 2 respiration) and decreased the efficiency of phosphorylation (a drop in the ADP/O ratio), but the effect of hyperthermia on state 3 respiration to a certain extent was dependent on the origin of mitochondrial tissue (13–15).

In this study, the response of the inner membrane of heart and liver mitochondria to hyperthermia in the range of febrile and supraphysiological temperatures was examined aiming to establish the pattern of the changes in the membrane barrier function, taking into account its crucial importance both for the efficiency of the oxidative phosphorylation and possible role in apoptotic or necrotic events. The data obtained by direct heating of isolated mitochondria were validated by experiments on the perfused liver.

Material and Methods

Animals. In the experiments, 3- to 4-month-old male and female Wistar rats weighing 275–300 g were used. The animals were acclimated to 22°C and a 12-hour light-dark cycle (lights on at 8 AM) and had free access to water and a standard chow diet.

Isolation of Mitochondria. Mitochondria were isolated from the heart and liver as described elsewhere (16). 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). The heart/liver was quickly removed and placed into ice-cold 0.9% KCl solution. The tissue was cut into small pieces and homogenized in a glass-Teflon homogenizer. Heart homogenization medium contained 160 mM KCl, 20 mM Tris, 10 mM NaCl, 5 mM EGTA, and 1 mg/mL bovine serum albumin (BSA) (pH 7.7); liver homogenization medium contained 10 mM Tris-HCl, 250 mM sucrose, 3 mM EGTA, and 2 mg/mL BSA (pH 7.7). The homogenate was centrifuged at 750*g* for 5 minutes, and the supernatant was centrifuged at 6800*g* for 10 minutes. The heart mitochondrial pellet was resuspended in suspension buffer containing 180 mM KCl, 20 mM Tris, 3 mM EGTA (pH 7.3) and stored on ice. Liver mitochondria were resuspended in suspension buffer containing 250 mM sucrose and 5 mM Tris-HC (pH 7.3) and centrifuged at 6800*g* for 10 minutes. The liver mitochondrial pellet was resuspended in liver suspension buffer. The protein concentration was determined by the biuret method (17) using BSA as a standard.

Liver Perfusion. After prenarcosis with CO_2, rats were anesthetized with a combination of an intraperitoneal injection of sodium pentobarbital (40 mg/kg body weight) and intramuscular injection of ketamine (100 mg/kg body weight). The peritoneal cavity was opened with a cross-abdominal incision, and heparin (1.5 IU/kg) was injected to the inferior vena cava. The liver was mobilized. After the portal vein was cannulated, the inferior vena cava and the aorta were transected. The liver was perfused manually through the portal vein with 20 mL of Krebs-Ringer solution with EGTA (120 mM NaCl, 24 mM $NaHCO_3$, 20 mM glucose, 5 mM HEPES, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, and 0.1 mM EGTA, pH 7.4) in order to remove the blood and clots. The liver was removed and transferred into a thermostatic vessel and perfused with an oxygenated (95% O_2, 5% CO_2) Krebs-Ringer solution with EGTA at a flow rate of 7 mL/min for 7 minutes. The liver was perfused with a Krebs-Ringer buffer solution with Ca^{2+} (120 mM NaCl, 24 mM $NaHCO_3$, 20 mM glucose, 5 mM HEPES, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.4 mM $CaCl_2$, pH 7.4) for 8 minutes. In each experiment, the liver was perfused at the appropriate temperature (38°C, 40°C, 42°C, 45°C, 46°C, and 48°C) with a preheated buffer solution. Immediately after the perfusion, the liver tissue was placed into ice-cold 0.9% KCl and subjected for the isolation of mitochondria.

Measurement of Mitochondrial Respiration and Membrane Potential. Mitochondrial respiration and membrane potential ($\Delta\psi$) were measured at different temperatures (37°C–48°C±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 (Zimkus A, Vilnius University, Lithuania) allowing simultaneous monitoring of $\Delta\psi$ and mitochondrial respiration. For each incubation, the TPP^+-sensitive electrode was calibrated by small additions of a tetraphenylphosphonium chloride solution to a final concentration of 267 nM. $\Delta\psi$ was calculated from the distribution of TPP^+ using the Nernst equation and the TPP^+ binding correction factor of 0.16 μL/mg protein (16).

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). The excess of creatine kinase (0.1 mg/mL) was added to maintain steady state respiration (18). The experiments were performed using 5-mM pyruvate plus 5-mM malate as the respiratory substrate. Mitochondria (1 mg protein/mL) were incubated in the assay medium with the respiratory substrate (state 2) for 3 minutes before the state 3 respiration was initiated by the addition of 1 mM ATP. The rate of mitochondrial respiration in state 2 (V_2) and state 3 (V_3) were defined according to the conventional terminology (19).

Determination of Dissolved Molecular Oxygen Concentration. The concentration of molecular oxygen dissolved in the assay medium at different temperatures (37°C–48°C±0.1°C) was determined polarographically using a glucose oxidase-catalyzed reaction

between D-glucose and O_2 (20). pH of the medium was strictly controlled at each temperature (pH 7.2). The reaction was initiated by adding 4.7 U/mL of glucose oxidase followed by 100 nmol of glucose each minute until complete oxygen consumption. The concentration of dissolved oxygen was calculated from the obtained polarographic curves from the amount of glucose used for the reaction. The molar ratio coefficient of the reaction between D-glucose and O_2 was defined in the medium with a known concentration of dissolved oxygen (0.9% NaCl solution in deionized water; oxygen solubility, 420.5 μmol O/L at 37°C; 760 mm Hg, atmospheric pressure). Dissolved oxygen concentrations determined in the medium were 395, 379, 371, 364, 356, 349, 341, 333, 326, and 318 μmol O/L at 37°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, and 47°C, respectively.

Modular Kinetic Analysis. The mitochondrial oxidative phosphorylation system was conceptually divided into 3 functional modules, interacting through a linking intermediate, $\Delta\psi$ (20) (Fig. 1). Although the true linking intermediate is protonmotive force (Δp), it has been previously shown that the measurement of $\Delta\psi$ instead of Δp does not introduce a significant error in the determination of module kinetics since ΔpH (the second component of Δp) is small and does not change under our experimental conditions (21). $\Delta\psi$ is generated by the respiratory module (comprised of the substrate carriers, matrix dehydrogenases, and respiratory chain complexes) and consumed by the phosphorylation module (comprised of ATP synthase, adenine nucleotide translocator, and phosphate carrier) and the membrane leak module (comprised of the passive permeability of the mitochondrial inner membrane to protons and any cation cycling reactions).

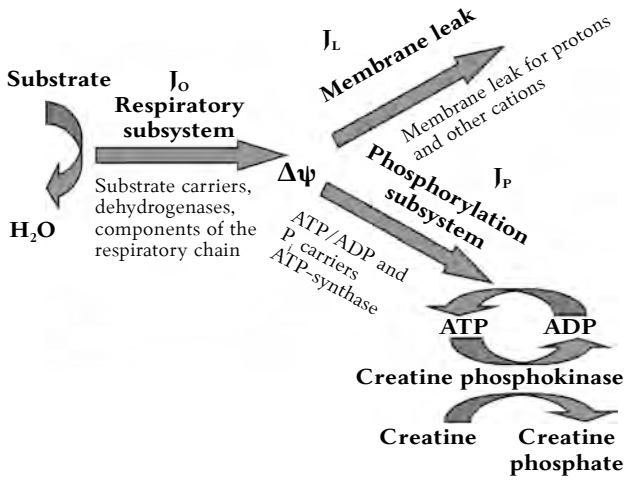

Fig. 1. Division of the oxidative phosphorylation system to 3 subsystems connected by the membrane potential $\Delta\psi$
J_O, flux through the respiratory subsystem, J_L, flux through the membrane leak subsystem, J_P, flux through the phosphorylation subsystem.

The dependence of the flux through the proton leak module (J_L) on $\Delta\psi$ 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 the addition of excess oligomycin (2 μg/mg mitochondrial protein). The experiments of modular kinetic analysis were paired for the heart and the unperfused liver: the measurements were performed at 37°C and 40°C or at 37°C and 42°C using the same mitochondrial preparation. The experiments of modular kinetic analysis were unpaired for the perfused liver: the proton leak was determined at 37°C for each 38°C–48°C perfused tissue mitochondrial preparation.

Results

The elucidation of molecular mechanisms of the response of both tumor and healthy cells to moderate heating is important in understanding the processes in cells subjected to fever-range hyperthermia or supraphysiological hyperthermia used for therapeutic purposes. The effect of hyperthermia on the mitochondrial membrane permeability of healthy tissue (heart and liver) mitochondria was evaluated using 2 modes of heating: 1) direct heating of the suspension of isolated mitochondria at appropriate temperature, or 2) hyperthermic (liver) perfusion and measurement of mitochondrial parameters at 37°C. In the direct heating model, the gender dependence using mitochondria isolated from the heart or liver of male and female animals was evaluated. Bearing in mind that the rate of mitochondrial respiration in metabolic state 2 (V_2) is almost entirely determined by the membrane permeability (16), the membrane permeability was evaluated by the measurement of V_2 (Fig. 2) and in some cases, when an increase in V_2 may be masked by inhibition of the respiratory module, by performing modular kinetics analysis.

Direct Heating of Heart Mitochondria. We performed the experiments of direct heating in vitro on mitochondria isolated from male and female rat hearts respiring with pyruvate plus malate as an oxidizable substrate. The obtained results indicated that an increase in temperature from 37°C to 40°C had no effect on respiratory rate in state 2 (V_2) in isolated rat heart mitochondria (Fig. 2 and Table 1). A further increase in temperature above 40°C led to the progressive increase in V_2 by 14% at 41°C and 63% at 42°C, while at 45°C, V_2 was more than 5-fold higher as compared to 37°C, implying strong uncoupling of oxidative phosphorylation due to increase in the membrane leak. There were no gender-dependent differences in temperature effects on the state 2 respiration in heart mitochondria (data not shown).

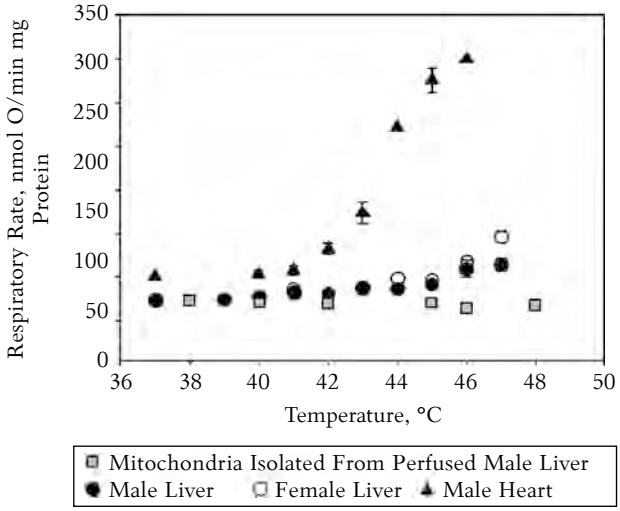

Fig. 2. The dependence of mitochondrial respiratory rate in state 2 on temperature

Averages from 3–6 independent experiments. Error bars represent SEM.

Table 1. Effect of Temperature on Male Rat Heart Mitochondria Respiration in State 2

Temperature °C	V_2, nmol O·min^{-1}·mg^{-1} Protein, mean±SEM	Stimulation as Compared With 37°C, %
37	51±1	–
40	54±4	6
41	58±5	14*
42	83±6	63*
45	275±14	439*

Substrate, pyruvate (5 mM) + malate (5 mM).
Averages from 3–6 independent experiments.
*Statistically significant temperature effect compared with 37°C (P<0.05).

In order to compare the effects of fever-range hyperthermia (40°C) and supraphysiological hyperthermia (42°C) on the membrane leak subsystem, modular kinetic analysis was used (Fig. 3).

The results showed that an increasing temperature from 37°C to 40°C had a negligible effect on the kinetics of proton leak. A further increase in temperature up to 42°C resulted in a remarkable increase in the flux through the membrane leak module (fluxes were compared at the same Δψ value). Comparing the fluxes through the membrane leak module at 140 mV (the value close to mitochondrial membrane potential in metabolic state 3, when mitochondria intensively phosphorylate ADP), one can see that the flux through the membrane leak module increased by 3.3-fold.

Direct Heating of Liver Mitochondria. Analogous experiments were performed with mitochondria isolated from male and female rat liver respiring with pyruvate plus malate as an oxidizable substrate. V_2 of isolated rat liver mitochondria was similar at 37°C for both genders, and for both genders, it

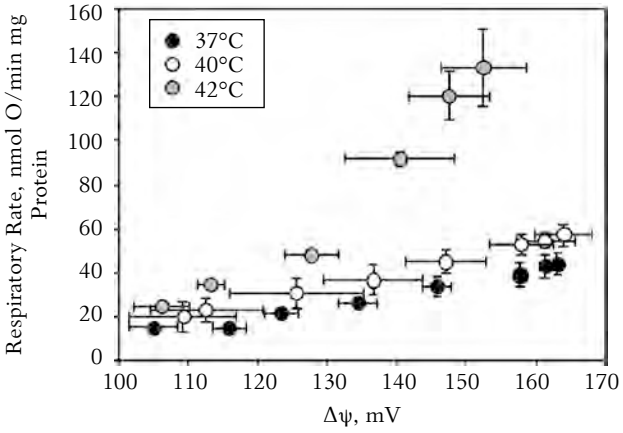

Fig. 3. The dependence of the membrane leak module kinetics on temperature in heart mitochondria

Oxidizable substrate, pyruvate + malate. Averages from 3–4 independent experiments. Error bars indicate SEM.

was significantly more sensitive to fever-range hyperthermia (40°C) in comparison with heart mitochondria (Figs. 2 and 3, Tables 2 and 3). However, with a further increase in temperature, V_2 in liver mitochondria from both genders did not increase so strongly as in heart mitochondria. Some tendency for a gender-dependent difference may be noticed at 45°C: V_2 was by 83% and 64% higher in female and male mitochondria, respectively, as compared with 37°C, although there was no statistical significant difference between V_2 in male and female

Table 2. Effect of Temperature on Female Rat Liver Mitochondria Respiration in State 2

Temperature °C	V_2, nmol O·min^{-1}·mg^{-1} Protein, mean±SEM	Stimulation as Compared With 37°C, %
37	24±1	–
40	28±1	17*
42	31±1	29*
45	44±3	83*
47	97±6	304*

Substrate, pyruvate (5 mM) + malate (5 mM). Averages from 3–6 independent experiments.
*Statistically significant temperature effect compared with 37°C (P<0.05).

Table 3. Effect of Temperature on Male Rat Liver Mitochondria Respiration in State 2

Temperature °C	V_2, nmol O·min^{-1}·mg^{-1} Protein, mean±SEM	Stimulation as Compared With 37°C, %
37	25±1	–
40	29±2	16*
42	32±2	28*
45	41±3	64*
47	65±7	160*

Substrate, pyruvate (5 mM) + malate (5 mM). Averages from 3–6 independent experiments.
*Statistically significant temperature effect compared with 37°C (P<0.05).

mitochondria at this temperature. This tendency became clear and significant at 47°C: V_2 was more than 4-fold higher in female and 2.6-fold higher in male mitochondria as compared with 37°C.

Hyperthermic Liver Perfusion. An additional experimental model system used to study hyperthermia-induced changes in membrane leak and possible recovery after heat stress employed mitochondria isolated from the liver subjected to hyperthermic perfusion with 38°C–48°C buffers for 15 minutes (V_2 was measured at 37°C). The results showed that V_2 stayed constant for all perfusion temperatures (Fig. 2). In order to resolve whether a short 15-minute hyperthermic perfusion had no effect on mitochondrial membrane permeability (or uncoupling took place but was reversible), or induced permeability changes of the inner mitochondrial membrane were masked by the inhibition of respiratory subsystem components, the effect of perfusion temperature on the kinetics of the membrane leak module was determined. One may see (Fig. 4) that a relatively short perfusion at 40°C and 42°C had no effect on membrane permeability (the obtained kinetics curves overlap). However, starting from 46°C, the respiratory rate (at the same membrane potential) substantially decreased, and this decrease was even more marked at 48°C. These results indicate that hyperthermic liver perfusion did not increase V_2 as compared with the direct heating of isolated liver mitochondria (Fig. 1) due to the inhibition of the components of the respiratory module.

Discussion

The results of the performed study has revealed that the inner membrane of mitochondria from healthy tissues is not less sensitive to hyperthermia

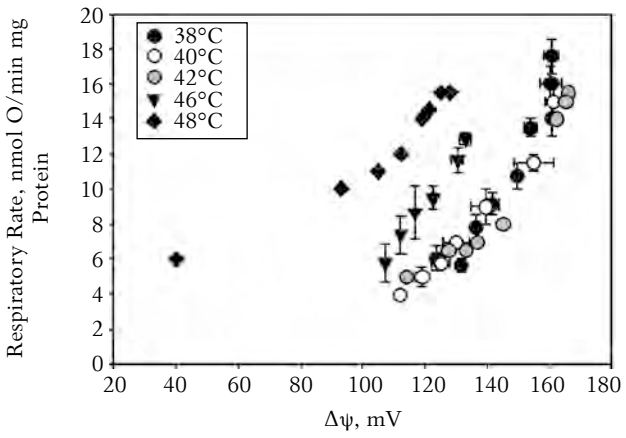

Fig. 4. The dependence of the membrane leak module kinetics in mitochondria isolated from perfused male liver on perfusion temperature

Oxidizable substrate, pyruvate + malate. Averages from 3 independent experiments. Error bars indicate SEM.

than plasma membrane that was reported to undergo very slight observable changes starting from 42°C (10). Our study demonstrated that the inner mitochondrial membrane might be sensitive even to fever-range hyperthermia; however, the response was tissue-specific – 40°C temperature did not induce statistically significant changes in the permeability of the membrane in isolated heart mitochondria (Table 1), whereas small but significant changes were observed in mitochondria isolated from the liver of both genders (Table 2 and 3). A comparable change in heart mitochondria was achieved at 41°C. Direct heating exceeding physiological hyperthermia (above 41°C) induced a very different response in heart and liver mitochondria (Fig. 2): a progressive and strong increase in the membrane permeability (up to complete uncoupling at 45°C) of heart mitochondria and a very moderate increase in V_2 in liver mitochondria that was more pronounced only at 45°C–47°C temperature. Our results also revealed that mitochondria isolated from female rat liver were more sensitive to severe heating than male rat mitochondria. That might be explained by gender-dependent differences in the composition and structure of the inner membrane. A very different pattern of the response of the membrane leak module in heart and liver mitochondria to the supraphysiological heating is due to the essential differences in temperature-induced effects on their respiratory subsystems. Our previous study has demonstrated that the respiratory subsystem in heart mitochondria is not very sensitive to hyperthermia (22) that even activates the respiratory chain components in the febrile range and only slightly inhibits the respiratory chain at higher temperatures (42°C–45°C). In contrast, the respiration of liver mitochondria in state 3 was strongly decreased by hyperthermia in the range from 43°C to 45°C, indicating a remarkable inhibition of the respiratory subsystem (23). This effect may entirely mask an increase in the membrane permeability in the presented experiments (Fig. 2).

Aiming to validate the effects of hyperthermia on the mitochondrial inner membrane observed in experiments in vitro by direct heating of isolated mitochondria, we also evaluated whether hyperthermia also affected membrane properties in mitochondria that were isolated from the liver subjected to short hyperthermic perfusion (Fig. 4). The estimation of the membrane leak module kinetics allowed us to confirm that more severe hyperthermia (46°C–48°C) induced a significant increase of the inner membrane permeability in male liver mitochondria from the perfused tissue, in accordance with the results of direct heating of mitochondria that are shown in Fig. 3 and Table 3. Possibly, more minor changes induced by the relatively short pe-

riod of milder hyperthermia (40°C–42°C) were not clearly visible or/and reversible.

Thus, our study has revealed for the first time that inner mitochondrial membrane is a cellular component very sensitive to hyperthermic treatment – possibly it is even more sensitive than cellular plasma membrane that was claimed by other authors (10) to be the most sensitive and the most early responding component to hyperthermia among all cellular components. In addition, it was shown that the response profile and hyperthermia-induced changes in the barrier function of the inner mitochondrial membrane may be strongly dependent on the tissue, and for liver mitochondria, this response exerted some gender-dependent features.

Among all healthy tissues, brain, neural, and heart tissues are among the most sensitive tissues to mild and moderate hyperthermia (24, 25). The risk of easily induced thermal damages in vitally critical organs determine reservations for the use of hyperthermia; in some cases, for local applications, but in particular, for the whole-body hyperthermia. One of the possible reasons of greater thermal sensitivity of excitable tissues might be related to the performance of their specific function – transduction of neural impulse. This process entirely relies on the proper barrier function of plasma membrane that evidently collapses at supraphysiological temperatures (10). On the other hand, the generation of action potential demands various ion gradients produced by ATPases. These membrane-embedded enzymes are strongly affected by temperature-induced changes in the structure and properties of the membrane. In addition, the activity of ATPases is absolutely dependent on energy supply; thus, on ATP production in mitochondria. In the light of these facts, it was of interest to compare mitochondria from excitable (heart) and nonexcitable (liver) tissues by the response of their membranes to heating. Our data showed that the observed kinetic response of proton leak was obviously different in heart and liver mitochondria. However, this apparent difference does not actually depend on the direct response of the mitochondrial membrane itself to heating. It is more likely that it is determined by a much higher sensitivity of the respiratory module components in liver mitochondria in comparison with that in heart mitochondria. Therefore, we conclude that hyperthermic treatment is able to evoke energy crisis in both normal heart and normal liver tissues, but it operates in a tissue-specific way: increases membrane permeability and induces strong uncoupling in heart mitochondria and causes the inhibition of electron flow in the respiratory chain in liver mitochondria. The response of brain mitochondria remains to be determined in this way.

Conclusions

The obtained results demonstrate that the mitochondrial membrane as well as energy production in mitochondria is very sensitive to supraphysiological hyperthermia. Taking into consideration the importance of mitochondria in cellular energy metabolism, stress response, their decisive role for death or survival both of tumor and healthy tissue cells, our data imply a crucial importance of the evaluation of the tissue- and gender-specific differences while developing or improving the protocols for hyperthermic treatment or combinatory therapy.

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Statement of Conflict of Interest

The authors state no conflict of interest.

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