

**Pathomechanism and therapeutic possibilities of mitochondrial dysfunction
in ischemia-reperfusion**

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List of full papers relating to the subject of the thesis

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Strifler G, Mészáros A, Pécz D, Ficzeré Á, Baráth B, Boros M, Hartmann P. A májfunkció vizsgálata respirometriával. MAGYAR SEBÉSZET (*accepted for publication*)

Abstracts relating to the subject of the thesis

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Strifler G, Fehér Á, Kaszonyi E, Gowda A, Kaszaki J, Boros M, Hartmann P. Metánlélegeztetés májmitokondriumokra gyakorolt hatása részleges máj ischaemia-reperfúziós károsodásban patkányokban. MAGYAR SEBÉSZET 68:(3) p. 115. (2015)

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1. INTRODUCTION

1.1. Physiological role of mitochondria

Mitochondria are membrane-enclosed organelles, the energy-producing centers in eukaryotic cells. The inner mitochondrial membrane is loaded with the protein complexes of the electron transport chain (ETC) and adenosine triphosphate (ATP) synthesis. This membrane surrounds the matrix, in which the citric acid cycle produces the electrons that enter and skip through the ETC. The outer membrane has many protein-based pores that allow the passage of ions and molecules as large as a small protein. Mitochondria produce ATP during oxidative phosphorylation, control the oxidative state of the cell, and are major regulators of caspase-dependent and caspase-independent apoptotic pathways (Li 2012).

1.2. Ischemia-reperfusion (IR)-induced mitochondrial disturbances

IR injury is a common complication of inflow-controlled major surgical resections and organ transplantations. The main source of reactive oxygen species (ROS) in hepatic IR injury are the mitochondria of activated sinusoidal endothelial cells and hepatocytes (Kalashnyk 2012). The lack of oxygen during ischemia causes a decrease in ATP production, and an increase in ATP hydrolysis due to the subsequent activation of anaerobic metabolism. In contrast, restoration of the blood flow during the reoxygenation phase leads to the overproduction of superoxide, mainly at the sites of complexes I and III (Kalashnyk 2012). Loss of calcium homeostasis, the production of ROS and the ETC damage are all different aspects of mitochondria-related changes in IR (Hines 2003).

1.3. Examination of mitochondrial functions with high-resolution respirometry

Respirometry detects the consumption of oxygen and it is the main tool to study mitochondrial function (Gnaiger 2000). The Oxygraph-2k (Oroboros Instruments, Innsbruck; Austria) is a second generation respirometer, equipped with two experimental chambers in which mitochondria, living cells or freshly prepared tissue samples are suspended in a suitable medium. Each chamber is supplied with an oxygen sensor and the oxygen consumption of mitochondria can be computed based on the changes in oxygen concentration in the chambers. During the respirometric measurements, the mitochondria undergo different “states” by the sequential addition of substrates or inhibitors.

1.4. Gas mediators and methane (CH₄)

Gaseous molecules, including nitric oxide, carbon monoxide and hydrogen sulfide have been known to play important roles in the biological systems. These gases were previously considered biologically inert, but recently have been found to be endogenously generated in the human body and mediate signalling pathways as biological messengers, and have been shown to have their own molecular targets. CH₄, the simplest alkane, is a novel candidate among therapeutic gases. At room temperature and at atmospheric pressure, CH₄ is a colorless, odorless gas. It is a simple asphyxiant, which means that tissue hypoxia may occur when CH₄ displaces the air and hence the oxygen in a restricted space and the concentration of oxygen is reduced to below approximately 18% in the internal milieu of the body). Information on the effects of exogenous CH₄ is sparse, but previous studies have demonstrated that CH₄ supplementation is beneficial against the deleterious effects of IR injury (Boros 2012). Other data also point to an anti-inflammatory and anti-apoptotic potential for CH₄, but the identification of intracellular targets remains elusive (Song 2015, Wu 2015, Ye 2015, Chen 2016, He 2016). We postulated that as critically involved in hypoxia-reoxygenation-induced intracellular respiratory damage, mitochondria may be targets of CH₄ administration. In particular, we hypothesized that if CH₄ is bioactive, it can exert its effect by influencing the respiratory activity and ROS production of mitochondria.

1.5. Biological effects of L-alpha glycerylphosphorylcholine (GPC)

GPC is a water-soluble deacylated metabolite of phosphatidylcholine (PC) (Brownawell 2011), a source of choline and a precursor of acetylcholine (Abbiati 1993; Parnetti 2001). The possible anti-inflammatory and scavenging potential of GPC is of particular importance, because it could offer means of targeting the inflammatory cascade without the confounding effects of mediators deriving from the metabolism of the lipid side-chains. GPC administration has been demonstrated to provide protection in neuroinflammation and restore ATP levels in the liver (Tókéš 2015). Furthermore, GPC administration can reduce several signs of oxidative and inflammatory tissue damage in experimental IR models (Hartmann 2014).

2. AIMS

- The primary goal was to characterize the functional changes of liver mitochondria in response to IR by means of high resolution respirometry.
 - First, we designed *in vitro* tests using intact liver mitochondria to study the mitochondrial functions directly.
 - Furthermore, we planned to investigate the mitochondrial ETC changes in the unstressed animals or after a standardized IR insult.
- Secondly, we set out to investigate the effects of potentially effective treatments on IR-damaged hepatic mitochondria.
 - We hypothesized that inhaled CH₄ can influence the mitochondrial respiratory activity.
 - We hypothesized that the protective mechanism of exogenous GPC is also linked to mitochondria.
- Thirdly, we set out to investigate the mechanism of action of inhaled CH₄ and GPC treatments; we hypothesized that these pathways will interfere with ROS generation caused by ETC dysfunction, and thus the pro-inflammatory cellular activation can be reduced.

3. MATERIALS AND METHODS

The experiments were carried out on male Sprague-Dawley rats (average weight 300±20 g, 7-8 weeks old) housed in an environmentally controlled room with a 12-hour light-dark cycle, and kept on commercial rat chow and tap water *ad libitum*. The experimental protocol was in accordance with EU directive 2010/63 for the protection of animals used for scientific purposes, and it was approved by the National Scientific Ethical Committee on Animal Experimentation (National Competent Authority) with the license number V./148/2013. This study also complied with the criteria of the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

3.1. Experimental groups and protocols

The rats were anesthetized with sodium pentobarbital (45 mg/kg ip), and the trachea was cannulated to facilitate respiration. The right jugular vein and carotid artery were cannulated

for fluid and drug administration, respectively. Supplementary doses of pentobarbital were given intravenously (iv) when necessary.

In the experimental series to study the effects of IR on liver mitochondria, complete ischemia of the median and left hepatic lobes was achieved by clamping the left lateral branches of the hepatic artery and the portal vein with a microsurgical clip for 60 min (IR group). After the period of ischemia, the clips were removed and measurements were performed during a 60-min reperfusion period. Sham-operated animals (SH group) underwent the same surgical procedure but liver ischemia was not induced.

In the experimental series to study the effects of CH₄ on liver mitochondria, the animals were randomly assigned to four groups. In the IR group, the rats were subjected to a 60-min complete ischemia followed by a 60-min reperfusion. Inhalation of 2.2% CH₄ in normoxic artificial air (Linde Gas, Hungary) was started after 50 min of ischemia which continued throughout the reperfusion period (IR+CH₄ group). The sham-operated animals in the SH group underwent the same surgical procedure but liver ischemia was not induced and the animals inhaled normoxic air, while the sham-operated animals in the SH+CH₄ group were likewise not subjected to liver ischemia, but inhaled the CH₄-air mixture for the same duration as in the IR+CH₄ group. Liver samples were taken from the affected lobes at the end of the reperfusion period and at identical time point from the sham-operated animals.

In the experimental series to study the effects of GPC on mitochondrial dysfunction-caused ROS generation the animals were randomly assigned to four groups. In the vehicle-treated IR group, the rats were subjected to 60-min complete ischemia which was followed by 60-min reperfusion. In the IR+GPC group 16.56 mg/kg bw GPC was injected iv and the same protocol was used 5 min before the end of ischemia. The sham-operated, vehicle-treated animals (SH group) underwent the same surgical procedure without liver ischemia, while another control group (SH+GPC group, n=6) received GPC in the same time-frame as the IR+GPC group.

3.2. High-resolution respirometry

Intact liver mitochondria from sham-operated animals were used to study *in vitro* mitochondrial functions directly. The isolation protocol was performed by the method of Gnaiger et al. For the *in vivo* experiments, liver biopsy samples were taken at different time point of the experimental protocols. Specific substrates, inhibitors and uncouplers (SUIT) of

the respiratory complexes were employed to investigate changes in the electron transport system (ETS). Glutamate (2 mM) and malate (10 mM) were used in combination to induce complex I-linked respiration; saturating ADP (2.5 mM final concentration) was added in order to stimulate respiration to the level of OxPhos capacity. By adding succinate (10 mM), the complex I+complex II OxPhos capacity was detected, then the uncoupler carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) (C; 0.5 μ M per step) was titrated. Finally, complex I was inhibited by rotenone (0.5 μ M) and complex III by antimycin A (2.5 μ M). In order to determine the leak respiration, the complex II-linked state II respiration rate was determined with 10 mM succinate, after the addition of 0.5 μ M complex I inhibitor rotenone. To determine the complex II-linked state III respiration, 2.5 mM ADP was added to each chamber. Finally, the leak respiration is measured in the leak state by inhibition of ATP synthase by adding 0.5 μ M oligomycin to the medium (state IV respiration).

3.3. Biochemical investigations

The cytochrome c oxidase activity was calculated via the time-dependent oxidation of cytochrome c at 550 nm (Szarka 2004). The activity of XOR was determined in the ultrafiltered supernatant by fluorometric kinetic assay based on the conversion of pterine to isoxanthopterin in the presence (total XOR) or absence (XO activity) of the electron acceptor methylene blue (Beckman 1989). The NADPH oxidase activity of the liver homogenates was determined by a modified chemiluminometric method of Bencsik et al. (Bencsik 2010). MPO activity was measured in liver biopsies by the method of Kuebler et al. (Kuebler 1996). The whole-blood superoxide and hydrogen peroxide (H₂O₂) production was measured with chemiluminometric method (Ferdinandy 2000). The lipid peroxide MDA level was measured through the reaction with thiobarbituric acid, by the method of Placer et al (Placer 1966) and corrected for the tissue protein content. The levels of NO_x, the stable end products of NO in the tissues were measured by the Griess reaction (Purnak 2012). Reduced glutathione and oxidized glutathione disulfide ratio (GSH/GSSG) in liver homogenates was determined by using a Fluorimetric Glutathione Assay Kit (Zitka 2012). *In vivo* histology was performed by means of laser-scanning confocal microscopy using fluorescent labelling (Goetz 2011). Apoptosis was detected by using the TUNEL/DAPI staining method.

3.4. Statistical analysis

Data analysis was performed with SigmaStat statistical software (Jandel Corporation, San Rafael, CA, USA). Changes in variables within and between groups were analyzed by two-

way repeated measures ANOVA, followed by the Bonferroni test in the cases of the mitochondrial respiratory function, the cytochrome c release from the mitochondria and whole-blood superoxide and H₂O₂ production; one-way ANOVA followed by the Holm-Sidak test was applied in the assay of tissue MDA, XOR activity, MPO activity, NADPH-oxidase activity, tissue nitrite/nitrate, H₂O₂ level and superoxide level. Data were expressed as means \pm SEM.

For statistical analysis of TUNEL and DAPI staining, the Kruskal-Wallis and Dunn tests were applied. Histological data were expressed as median \pm SD. Values of $P < 0.05$ were considered statistically significant.

4. RESULTS

4.1. IR-induced mitochondrial dysfunctions

In response to IR injury, both complex I and II basal respiration decreased, as compared with the sham-operated animals, which refer to a lower capacity of the ETS (120.7 ± 6.2 pmol/ml/sec vs 80.6 ± 2.8 pmol/ml/sec). The ADP-stimulated oxidative phosphorylation capacity was also decreased at the end of ischemia and throughout the reperfusion phase. Coupling of mitochondrial respiration was significantly damaged in the IR group (140.9 ± 65.9 pmol/ml/sec vs 90.2 ± 3.09 pmol/ml/sec), in parallel with the increased leak respiration, which is a compensatory mechanism for the proton leak at a high chemiosmotic potential, when ATP synthase is not active. Increased leak respiration in the IR group refers to mitochondrial membrane-damage and mitochondrial ROS production. These processes directly lead to increased ROS production. Upon application of selective inhibitors of the mitochondrial complexes the complex I-linked respiratory activity was significantly reduced in the IR group as compared to the control group (70.77 ± 2.39 pmol/ml/sec vs 40.9 ± 2.05 pmol/ml/sec). The above data provided evidence for the major role of complex I in IR injury-related mitochondrial dysfunction

4.2. Effects of CH₄ on mitochondrial function

The complex II-linked respiratory flux values were significantly lower in the IR group at 55 min of ischemia and at 60 min of reperfusion than those of the SH animals. The IR-induced decreases in basal flux were reversed in response to CH₄ treatment. Interestingly,

CH₄ treatment alone (SH+CH₄), elevated the basal oxygen consumption throughout the observation period.

In comparison with SH, IR resulted in a lower oxidative phosphorylation capacity of the mitochondria (complex II-linked state III respiration) throughout the examination period. When CH₄ inhalation was applied, however, the respiratory capacity was preserved at 55 min of ischemia and at 30 min of reperfusion (55.7±8.2 pmol/ml/sec vs 82.6±5.8 pmol/ml/sec). The cytochrome c oxidase activity was also determined with a spectrophotometric analysis. In comparison with the SH group, the IR group exhibited significantly higher cytochrome c oxidase activities during the reperfusion period, as an indication of functional damage. In the IR+CH₄ group, the cytochrome c release did not increase in response to the IR-induced damage.

4.3. Effects of GPC on mitochondrial function

In vitro experiments were conducted in order to analyse the dose-response effects of GPC on the respiratory activity of rat liver mitochondria in normoxia or anoxic conditions. GPC had an increasing effect on mitochondrial oxygen consumption in the 100-200 mM concentration ranges. The ETC and oxidative phosphorylation capacity of mitochondria was influenced significantly when GPC was applied at 200 mM concentration (160.8±12.2 pmol/ml/sec vs 390.7±24.8 pmol/ml/sec). In addition, GPC significantly attenuated the deleterious effects of 30-min anoxia on the oxygen consumption of liver mitochondria .

The effects of GPC on IR-induced mitochondrial dysfunction *in vivo* were evaluated with the SUIT protocol. The state III oxygen consumption was significantly lower in IR group as compared to the sham-operated animals. Additionally, the maximum respiratory capacity was also significantly lower in response to the IR stress. In contrast, treatment with GPC enhanced the efficacy of oxygen consumption. These effects were basically linked to the complex I, rather than complex II, as indicated by the large decrease following the administration of the inhibitor of complex I.

4.4. Effects of CH₄ on oxidative damage and structural changes

The whole-blood superoxide-producing capacity was significantly higher in the IR group at 30 min of reperfusion in comparison with the SH animals. The CH₄ inhalation before the end of the ischemic period reduced the elevated superoxide production to the level in the SH animals. Significantly higher whole-blood H₂O₂ levels were measured at 5, 30 and 60 min of

reperfusion in the IR group relative to the SH group. The CH₄ inhalation protocol effectively reversed the H₂O₂ production in the IR+CH₄ group. A significantly higher MDA level was measured at the end of reperfusion in the IR group as compared to that in the SH group. The IR-induced elevation of the liver MDA level was effectively attenuated by CH₄ inhalation in the IR+CH₄ group.

The morphological changes in the left liver lobe were evaluated by means of *in vivo* imaging, using confocal laser scanning endomicroscopy. The intravital FITC-dextran and acriflavine staining demonstrated more dilated sinusoids in the IR group, and also histological signs of apoptosis: a loss of fluorescence intensity, changes in hexagonal cell shape and cytoplasm blebbing and vesicle formation relative to the SH group. CH₄ inhalation effectively attenuated these apoptosis-linked morphological changes.

As expected, only a few TUNEL-positive cells was observed in the liver specimens of the rats in the SH+CH₄ and SH groups after conventional histology analysis. Conversely, liver IR was accompanied by an increased TUNEL positivity, which was diminished as a result of CH₄ inhalation (IR and IR+CH₄ groups).

4.5. Effects of GPC on oxidative damage

In this study, XOR activity was increased in the IR group as compared to the SH group. These values were significantly decreased when GPC was applied 5 min before the reperfusion. By the end of the 60-min reperfusion period, the NADPH oxidase activity was significantly increased in the IR group, compared to the SH groups. When GPC was administered before the end of ischemia the NADPH oxidase activity became even lower than the values of the SH groups. In the IR group, the tissue MPO level was significantly increased as compared with that of the sham-operated animals. In the IR+GPC-treated group, the MPO activity was significantly lower than in the IR group. The superoxide-producing capacity in the whole blood was significantly higher in the IR group at the end of reperfusion when compared to the SH animals. The GPC treatment before the end of the ischemic period reduced the elevated superoxide production to the level in the control animals. Significantly higher whole blood H₂O₂ levels were measured at the end of reperfusion in the IR group relative to the SH group, and the GPC treatment effectively reversed the H₂O₂ production. As expected, IR resulted in an increased MDA production after IR. The GPC treatment significantly reduced the level of MDA production, while no difference was seen between the two control groups (SH and SH+GPC). In the IR group, a significant elevation in NO_x was

present relative to the SH groups. The GPC treatment protocol decreased the NO_x elevation, in contrast with the non-treated IR group; but the NO_x level remained significantly higher than that in the sham-operated group. GPC administration in the SH+GPC group did not influence the GSH/GSSG ratio as compared with the SH group.

The hepatocytes were exposed to increased levels of oxidative stress after IR, as evidenced by a significant increase of GSSG and decreased GSH/GSSG ratio when compared to the SH group. However, the GSSG levels were significantly decreased in response to GPC treatment in the IR+GPC group.

The *in vivo* histology of the liver have demonstrated dilated sinusoids and fluorescent dye leakage with edema formation. Visible signs of structural damage were present in the IR group: changes in hexagonal cell shape and cytoplasm blebbing and vesicle formation. GPC administration effectively attenuated the IR-induced morphological changes.

5. DISCUSSION

5.1. IR-induced changes in mitochondrial functions

In response to IR-injury, the basal respiratory activity of mitochondria was significantly decreased, which refers to a lower capacity of the respiratory chain to transmit electrons toward the ATP synthase. The ADP-stimulated oxidative phosphorylation capacity was also decreased at the end of ischemia and throughout the reperfusion phase. The application of selective substrates and inhibitors of the mitochondrial complexes provided evidence for the major role of complex I in IR injury, as demonstrated by decreased oxygen consumption and the increased leak respiration.

5.2. Effects of CH₄ in mitochondrial dysfunction

We have investigated the *in vivo* influence of an increased CH₄ intake on the respiratory activity of rat liver mitochondria by using controlled ventilation. The inhalation of 2.2 % CH₄-containing normoxic artificial air preserved the oxidative phosphorylation after a period of tissue ischemia, and significantly improved the basal mitochondrial respiration state after the onset of reperfusion. In line with this, the IR-induced ROS production, cytochrome c release and hepatocyte apoptosis were also significantly reduced.

During ischemia, the mitochondrial NADH/NAD⁺ and FADH/FAD⁺ ratios remain elevated, leading to reductive stress (Boros 1999; Ghyczy 2008), while reperfusion of the previously ischemic tissue leads to oxidative stress with a burst of ROS generation following the start of reoxygenation. It was earlier suggested (without indicating the exact biochemistry, contributing compounds or enzymes) that ROS reactions could lead to a higher level of fixation of CH₄ in a lipid environment, such as the mitochondrium membrane (Carlisle 2005; Dougherty 1967). Indeed, IR perturbs the heterogeneous lipid-bilayer membrane structure and changes the fluidity from fluid to gel. Disordered/fluidized bilayer states could therefore be analogous to physical damage to the ETC in these conditions. We have shown that the levels of both ROS and MDA were reduced by an increased CH₄ intake, indirectly demonstrating the decreased oxidative damage to the mitochondrial membranes.

5.3. Effects of GPC on ROS production

The *in vitro* experimental data demonstrated the direct effects of GPC on mitochondrial oxygen consumption in the 100 and 200 mM concentration ranges. Next, GPC supplementation attenuated the respiratory consequences of anoxia by reducing the leak of protons into the matrix. This effect had mainly been attributed to an action on complex I at the appropriate concentration of 200 mM. Within the mitochondria, the mechanism by which GPC increases basal oxygen consumption rates is not well-understood, nevertheless, there are two plausible explanations: 1) by interacting with proteins and causing modulations in their functions or 2) by influencing the redox environment. Furthermore, the I-V sequence of the respiratory complexes is perhaps not the highest level of oxidative phosphorylation organization.

We have shown that exogenous GPC targets the mitochondrial oxidative metabolism in IR stress, and provided evidence that the IR-associated inflammatory activation may be limited this way. We have demonstrated that GPC treatment reduces the leak respiration after the IR challenge, and in accordance with previous findings the lower leak respiration was accompanied with decreased ROS formation. In response to complex I inhibitor rotenone, the oxygen consumption diminished significantly, which suggests that complex I is the target site of the GPC-mediated action.

We have investigated IR-induced ETC changes together with XOR and NADPH oxidase responses. The activity of both pro-inflammatory enzymes were decreased in response to GPC administration, which suggests that its primary influence on leak respiration was followed by

secondary consequences on the main extra-mitochondrial, i.e. cellular enzymes involved in ROS formation. Furthermore, the IR-induced increases in superoxide and H₂O₂ levels in the circulating blood were accompanied by increased local NO_x concentrations, providing indirect evidence for an evolving oxido-nitrosative stress in the liver tissue.

In our model, the increase in MDA and other oxidative and nitrosative stress markers were significantly reduced by GPC supplementation. The need of restoration of cellular GSH levels for efficient scavenging of peroxynitrite is emphasized. GPC administration reversed the IR-induced decrease in GSH level, and maintained the ratio of GSH to GSSG. We also detected increased MPO activity as a secondary inflammatory marker, mainly secreted by active immune cells including PMNs. Again, MPO activity was decreased after the administration of GPC. All considered, these results suggest that mitochondrial alterations preceded the cellular, enzymatic ROS production and the onset of oxidative stress in liver tissue lead to PMN activation in the circulation.

6. SUMMARY OF NEW FINDINGS

- Liver IR injury is a progressive process starting from a depressed mitochondrial ETS, then the abnormal formation of ROS leads to biomembrane damage, and finally to necrotic or apoptotic cell death. The mitochondrial protection afforded by CH₄ inhalation involves different components under normal conditions, during ischemia and during reperfusion, similarly to the different pathomechanisms of damage.
- 2.2% CH₄ inhalation significantly influenced the IR-related disturbances of the mitochondrial ETS and mitigated the severity of subsequent damaging events. The protective potential of CH₄ was linked to reduced cytochrome c release and a reduced number of apoptotic hepatocytes.
- Exogenous GPC influences the mitochondrial oxidative metabolism, the primary source of ROS production. The direct action of GPC on mitochondrial complex I function leads to increased oxygen consumption and reduced leak respiration.
- GPC administration attenuated membrane peroxidation and the subsequent stages of tissue damage, therefore this compound might be therapeutic in liver IR episodes.
- Both CH₄ and GPC treatments could effectively attenuate pro-inflammatory activation in IR stress through targeting the mitochondrial oxidative metabolism.

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