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THE PROTECTIVE ROLE OF METHANE IN MITOCHONDRIAL AND METABOLIC PATHWAYS OF ISCHEMIA/REPERFUSION-INDUCED CARDIAC DAMAGE

Ph.D. Thesis

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INTRODUCTION

Ischemia-reperfusion (I/R)

Cardiac muscle cell contraction is essential for maintaining the life-long need of blood flow in our body. This mechanism, however, requires loads of oxygen to work in proper way. Most common cardiac conditions (e.g. myocardial infarction, hypertrophy, etc.) involve ischemia (decrease in blood flow and consequential loss of tissue oxygen level) in their pathomechanism. Warm ischemic changes are built around the hypoxic intracellular milieu of cardiac muscle cells. Low intracellular oxygen level leads to mitochondrial deceleration, which causes adenosine-triphosphate (ATP) depletion. Disability of mitochondrial electron transport chain (ETC) also causes upstream accumulation of succinate (Succ) and nicotinamide adenine dinucleotide hydride (NADH), which activates several alternative metabolic pathways. ATP loss of the cell also realized in the diminished function of primarily or secondarily ATP dependent ion channels. Subsequent overdosage of oxygen and previously decelerated mitochondria together lead to production and release of reactive oxygen species (ROS). Cold ischemia is based on the same concept of hypoxia as warm ischemia, however, cold (4°C) environment decelerates metabolic rate of organs and tissues, so it can be used to induce effective protection against harmful metabolic changes. This characteristic makes cold ischemia suitable for organ preservation in transplantation. Cold ischemia also exhausts energy stores while ATP production is decreased and anaerobic metabolic pathways activate. Intracellular Ca^{2+} overload is taken up by the mitochondria, but remained high intracellular Ca^{2+} concentration further activates pro-apoptotic enzymes, such as phospholipases, calmodulin regulated proteases and endonucleases. Ca^{2+} is also released from the endoplasmic reticulum (ER) as a consequence of cold environment. Ca^{2+} not only provokes pro-apoptotic enzyme activity, it promotes changes in gene expression via the hypoxia-inducible factor 1-alpha (Hif-1α) pathway and even take part in the inner pathway of apoptosis by modulating mitochondrial death signals of Bax/Bcl2 system.

Mechanism of I/R-induced mitochondrial dysfunction

Avoiding mitochondria-connected damage of the graft should be considered as the main goal to improve organ preservation methods. Life of the cell is strongly attached to mitochondrial metabolism through mechanisms such as ATP synthesis or pro- and anti-apoptotic signaling pathways. I/R initiates organ dysfunction through intracellular hypoxic damage, which leads to unsuccessful graft transplantation. Absence of blood flow and consequential ischemia

causes metabolic shift into anaerobic glycolysis involving the reduction of electron transport through ETC. Hypoxia also inhibits the function of succinate dehydrogenase (respiratory complex II), thus leading to NADH and succinate accumulation; which is considered as a metabolic marker of ischemia. Under hypoxic condition, accumulated intracellular Ca^{2+} is proved to influence mitochondrial permeability, potentiating ROS production, both of which are enhanced during reperfusion. During reperfusion, the reaction of previously leaked electrons and highly accessible oxygen result in ROS accumulation, which leads to more pronounced ATP depletion, completing the vicious circle of I/R, eventually initiating apoptosis. Increased ROS disintegrates mitochondrial lipid membrane, which opens mitochondrial permeability transition pores (mPTPs) promoting Ca^{2+}-induced mitochondrial swelling.

Cardiac transplantation

In practice, transplantation involves both warm ischemic (during the surgical process) and cold ischemic (during cold storage) injury in its pathomechanism. Transplantation is necessary in the treatment of end-stage organ failure, however improving graft survival and providing patient safety is still in the focus of clinical sciences. Nowadays, static cold storage is still the most applied method for organ preservation after surgical explantation. Currently used techniques provide limited protection against transient anoxia or reperfusion-induced tissue damage, therefore, seeking for advanced methods to avoid cold storage-related organ dysfunction is highly researched.

Chemical properties and bioactivity of methane (CH_4)

CH_4 is a colorless, odorless, omnipresent gaseous molecule, while being inert at ambient temperature and pressure. In the upper layer of the atmosphere CH_4 plays a role in ozone formation by reacting with hydroxyl radicals, however, physico-chemical reactions of CH_4 in eukaryotic organisms are not fully discovered. In this line, proofs are available for the production of CH_4 as a result of aerobic metabolic processes, and besides, *in vivo* studies described the biological effects of exogenously administered CH_4. CH_4 inhalation or CH_4-enriched fluid therapies are proved to be protective against the consequences of I/R-induced conditions in intestinal and liver I/R injury. Furthermore, anti-oxidative, anti-apoptotic, and anti-inflammatory effects of CH_4 inhalation or CH_4-enriched fluid therapies have been demonstrated in inflammatory conditions and sepsis.

The effect of CH_4 on I/R injury

Mitochondria can be influenced by exogenously administered CH_4 in I/R injury *via* potential non-specific physico-chemical alterations of phospholipid bilayer of biological membranes.

I/R injury affects the ETC, which is embedded in the inner layer of mitochondrial membrane. Similar to other simple gaseous molecules, CH_4 is likely to exert site-specific action on mitochondrial proteins or protein complexes. It seems that complex IV (cytochrome c oxidase), one of the protein complexes of the mitochondrial ETC, may be responsible for the action of CH_4.

The role of mitochondria in I/R damage

Mitochondria are intracellular organelles, which function mainly is to provide energy for cell metabolism in the form of ATP. Also, mitochondria take part in several biochemical pathways of cellular metabolism, such as β-oxidation of fatty acids, Krebs cycle and alternative carbohydrate metabolic pathways. Although energy production through mitochondrial ETC is substantial in terms of the life of eukaryotic cells; however, oxidative phosphorylation of mitochondria can be responsible for not only the metabolism of the cell but also its death. Mitochondrial ETC can be the main character in developing reperfusion injury. Overcharging the ETC with oxygen in the reperfusion period leads excessive function of ATP synthase enzyme known as complex V, which is leading to acceleration of the electron transport creating electron leakage through different complexes. Leaked electrons can be redirected into the ETC, or can reduce NAD^+ causing intracellular NADH accumulation. Another possibility for decreasing the locally elevated electron levels is using the already high amount of oxygen in the system thus creating ROS which is the main source of oxido-reductive stress during reperfusion.

MAIN GOALS

We hypothesized that addition of CH_4 to a cold preservation solution modifies the I/R-damage of the graft. (Study I)

- Therefore, we examined the effect of CH_4–enrichment on the oxido-reductive and nitrosative stress pathways in the myocardium in a rat model of isogenic heart transplantation.
- We also investigated the expression of ER stress and mitochondrial apoptosis markers as possible underlying mechanisms of the cytoprotective action of CH_4 enrichment.

As a next step, we hypothesized that CH_4 influences the extramitochondrial Ca^{2+} streams, which play a major role in the ER—mitochondria interaction. (Study II).

- For this purpose, we investigated the effects of exogenous Ca^{2+} on the membrane potential changes of isolated cardiac mitochondria.
- Finally, we aimed to explore the potential target site on ETC responsible for the mitochondrial effects of CH_4 by using selective substrates and inhibitors of the respiratory complexes.

MATERIALS AND METHODS

Study I. Investigation of the effects of CH_4–enrichment of preservation solution on heart grafts in an experimental transplantation setup

The experiments were carried out on male Lewis rats (250–350 g; Charles River, Germany) in accordance with EU Directive 2010/63 for the protection of animals used for scientific purposes and in compliance with criteria set down in the US National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The study was approved by the national competent authority of Hungary (ATET) under licence number PEI/001/2374-4/2015.

Experimental protocol

Isogenic male Lewis to Lewis heterotopic transplantation (HTX) (n=36) was performed as described previously. Briefly, after excision from the donors, the grafts were cold stored in a transplantation solution for 60 min (cold ischemia time), which was followed by HTX and a 60-min reperfusion period. At the end of the reperfusion, hemodynamic measurements were performed to evaluate early graft functions; thereafter, biopsies were taken from the left ventricle (LV) of the grafts for mitochondrial functional measurements and biochemical assays. Tissue myeloperoxidase (MPO) and xanthine oxidoreductase (XOR) activity, reduced glutathione (GSH) and oxidized glutathione disulfide (GSSG) ratio, and tissue nitrite/nitrate (NOx) level were determined. Blood samples for serum biomarkers of myocardial injury were taken from the vena cava at the end of the reperfusion period.

The animals were randomly allocated into three groups. In group 1 (control, n=12), donor rats underwent the same surgical procedure until the explantation, but the hearts were not subjected to cold storage and were not transplanted. In group 2 (n=12), the explanted grafts were stored in CS solution at 4ºC during the 60-min cold ischaemic period, while the grafts were stored in CH_4-enriched CS during the cold ischaemic period in group 3 (n=12).

Rat model of heterotopic heart transplantation (HTX)

HTX was performed in isogenic Lewis rats to avoid organ rejection. The technique has previously been described in detail. Briefly, donor rats were anaesthetised with isoflurane, and heparin (25000 IU) was administered intravenously. A bilateral thoracotomy was performed to expose the heart and cold (4°C) cardioplegic CS solution (Dr. Franz Köhler Chemie GmbH, Bensheim, Germany, n=12) or CH_4-enriched CS (CS-CH_4, n=12) was infused into the coronaries via the aorta. The superior and inferior vena cava and the pulmonary veins were tied en masse with a suture, and the heart was excised with the aortic arch for the measurement of hemodynamics, including the coronary blood flow (CBF). After excision, the

grafts were stored either in cold CS or CS-CH_4 for 60 min. Control hearts underwent the same surgical procedure but were not subjected to cold storage and transplantation (Control, n=12). The recipient rats were anaesthetised with isoflurane and then heparinized (400 IU/kg iv), and body temperature was maintained at 37°C on a heating pad. Approximately 2-cm segments of the infrarenal aorta and the inferior vena cava were isolated and occluded with small-vessel forceps. The aorta and the pulmonary artery of the donor heart were anastomosed end to side to the abdominal aorta and the vena cava of the recipient, respectively. The duration of the implantation was standardized at 60 min (ischaemic period) to minimize time-related variability between experiments. After completion of the anastomoses, protamine (400 IU/kg iv) was administered to antagonize heparin effects. The occlusion was released, and the donor heart was reperfused with blood *in situ* for 60 min.

Hemodynamic measurements

60 min after transplantation, a 3F latex balloon catheter (Edwards Lifesciences Corporation, Irvine, CA, USA) was introduced into the left ventricle (LV) via the apex to determine the maximal LV systolic pressure (LVSP), dP/dtmax (maximal slope of systolic pressure increment) and dP/dtmin by a Millar micromanometer (SPR-838, Millar Instruments) at different LV volumes (20-180 µl). LV pressure-volume relationships were constructed from these data. The CBF of the graft was measured with an ultrasonic flow meter (Transonic Systems Inc., Ithaca, USA) mounted on the donor ascending aorta.

Examination of mitochondrial function

The efficacy of the mitochondrial respiration was assessed from tissue samples homogenized in 3 ml mitochondrial respiration medium (MiRO5) by high-resolution respirometry (HRR, Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria). Mitochondrial O_2 consumption (respiratory flux), complex II-linked baseline respiration (Succ-fuelled, in the presence of complex I inhibitor rotenone), oxidative phosphorylation capacity (OxPhos) and cytochrome c release (an indicator of inner mitochondrial membrane damage) were determined.

Quantitative real-time PCR (qPCR) analysis

Myocardial mRNA expression was analysed by qPCR (Applied Biosystems, Foster City, CA, USA) for the following genes: caspase-3, caspase-9, DNA damage-inducible transcript 3 (Ddit3, also known as CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP)), Hif1α, GSK-3β and very low-density lipoprotein receptor (VLDLr).

Biochemical assays

Tissue xanthine oxidoreductase (XOR) activity

Heart biopsies were homogenized in a phosphate buffer (pH 7.4). The homogenate was centrifuged at 4°C for 20 min at 24 000 g and the supernatant was loaded into centrifugal concentrator tubes. The XOR activity was determined in the ultrafiltered supernatant by a fluorometric kinetic assay based on the conversion of pterine to isoxanthopterine in the presence (total XOR) or absence (XO activity) of the electron acceptor methylene blue.

Reduced glutathione and oxidized glutathione disulfide (GSH/GSSG) ratio in heart homogenates

The GSH/GSSG ratio was determined using a Fluorimetric Gluthatione Assay Kit (Sigma Aldrich, Budapest, Hungary). The GSH content of the sample can be determined by quantifying the thiol concentration in biological samples by reacting with the thiol groups they contain. The adduct can be detected by fluorimetry at 478 nm. The GSSG content of the sample was calculated following the manufacturer's recommendations.

Tissue myeloperoxidase (MPO) activity

The MPO activity was measured in biopsies using a method developed by Kuebler at al. Briefly, the tissue was homogenized with Tris-HCl buffer (0.1 M, pH 7.4) containing 0.1 M polymethylsulfonyl fluoride to block tissue proteases, and then centrifuged at 4°C for 20 min at 24000 g. The MPO activity of the samples was measured at 450 nm (UV-1601 spectrophotometer; Shimadzu, Japan), and the data were referred to the protein content.

Heart nitrite/nitrate (NOx) levels

The levels of NOx, the stable end products of NO in the tissues were measured with the Griess reaction. This assay is based on the enzymatic reduction of nitrate to nitrite, which is then converted into a coloured azo compound, which is detected spectrophotometrically at 540 nm.

Histology and immunohistochemistry

The hearts were immersed and fixed in buffered paraformaldehyde solution (4%) embedded in paraffin, and 5-μm thick sections were stained with haematoxylin and eosin (H&E). Immunohistochemical staining for the ER stress marker sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA1) was performed with SERCA1-specific antibody (#S1189, Sigma Aldrich, Budapest, Hungary) as previously described. Histological assessment was performed independently and blindly using a previously described 0–4-grade histological scoring system, representing a composite of number of damaged myocytes and number of foci of damage. SERCA1 immunoreactivity was determined using open-resource imaging software (ImageJ version 1.8) and expressed as a percentage of immunopositive cells quantified per field of view.

Study II. *In vitro* effects of CH_4 during anoxic stress and evaluation of mitochondrial membrane potential changes

We examined the effects of dissolved CH_4 on isolated mitochondria of rat cardiac muscle cells under anoxia re-oxygenation (A/R).

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 Ethics Committee on Animal Experimentation (National Competent Authority) under 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.

Experimental protocol

Isolated cardiac mitochondria were treated as follows: anoxia was induced using 100% N_2 persufflation for 2 h into a 2 ml volume cuvette containing 1 ml respiratory medium and 1 ml airspace. Anoxia was followed by a re-oxygenation period (95% air and 5% CO_2) with or without 2.2% CH_4 supplementation for 30 min (the A/R and A/R+CH_4 groups, respectively) (n=12–16). In the control groups, the mitochondria were kept in normoxic cuvettes (95% air and 5% CO_2) with or without 2.2% CH_4 supplementation (the normoxia and normoxia+CH_4 groups, respectively). Then mitochondria were subjected to HRR.

Examination of mitochondrial functions

HRR was used to examine the oxygen consumption of the isolated cardiac mitochondria under stimuli of different mitochondrial complexes, to determine mitochondrial hydrogen peroxide (H_2O_2) production and to detect changes in mitochondrial membrane potential.

Mitochondrial H_2O_2 production

Mitochondrial H_2O_2 release as a marker of ROS (i.e. superoxide anion) production was monitored fluorimetrically with the Amplex Red/horseradish peroxidase system, whereby Amplex Red (non-fluorescent) is oxidised to Resorufin. H_2O_2 production was calibrated with known amounts of H_2O_2. In this setup, ROS release was investigated by adding oxidizing substrates (10 mM glutamate, 5 mM malate, 20 mM S, 5 mM ADP) to the mitochondria. On isolated mitochondria, the reverse electron transport (RET)-initiated H_2O_2 flux was measured when mitochondria were incubated with 20 mM Succ; it was then blocked by the addition of 1 μM rotenone (Rot). The residual oxygen consumption was estimated after addition of 1 μM antimycin A (Ama, an inhibitor of Complex III) to exclude the effects of oxidative side reactions. Then, free radical leak was also determined as the percentage of oxygen consumption diverted to the production of H_2O_2 in State 3 respiration.

Mitochondrial membrane potential

Mitochondrial membrane potential was measured fluorimetrically using the fluorophore agent safranin. First, we added 1μM Rot, 10mM Succ and 1μM CCCP. Finally, residual oxygen consumption (ROX) was determined by adding 1 μM Ama.

Extramitochondrial Ca^{2+} -induced membrane potential changes

Membrane potential changes evoked by extramitochondrial Ca^{2+} in normoxic and anoxic environment was assessed in isolated cardiac mitochondria using a blue fluorescence sensor (excitation 465 nm; gain for sensor: 1000 and polarization voltage: 500 mV) on a high-resolution fluorespirometer. After the four-step titration of safranin, 100μl of samples were loaded into the chambers. Then, mitochondria were treated with 1μl of 0.5 μM Rot and 20μl of 10 mM Succ. The responsiveness of the fluorescent dye was monitored by exogenous 20 μl of 20mM $CaCl_2$ in three-step titration until reaching a plateau. The Ca^{2+} signal was eliminated with the addition of 1 μl of 1 μM Ama. Ca^{2+}-related changes were expressed as the rate of changes in fluorescent signal and average resting fluorescence.

RESULTS

Hemodynamic parameters of the transplanted grafts

After transplantation, increasing LV balloon volumes ('preload') resulted in elevated LVSP and dP/dtmax, which were both significantly increased at the largest preload values in the CS-CH_4 group as compared to CS alone. A similar change in diastolic function was noted at higher preload volumes, bringing about significantly elevated dP/dtmin values ($p < 0.05$) compared to CS, reflecting better myocardial relaxation. CBF was also significantly ($p < 0.05$) higher after 60 min of reperfusion in CS-CH_4 storage as compared to the CS group. There was no statistically significant difference in HR values within and between experimental groups.

Cardiac mitochondrial function

Complex II-linked basal respiration was significantly higher in the CS-CH_4 grafts than in the CS group 60 min after reperfusion. After adding saturating amounts of ADP, the OxPhos was significantly higher in the CS-CH_4 group. Mitochondrial respiration in response to cytochrome c was tested to determine the ability of exogenous cytochrome c to replace the enzyme in the mitochondrial membrane. In comparison with the CS group, the release of cytochrome c was significantly lower in the CS-CH_4 group.

Myocardial ER stress- and apoptosis-associated gene expression

The relative mRNA expression for hypoxia- and ER stress-associated genes (Hif-1α, CHOP, GSK-3β and VLDLr) was significantly lower in the CS-CH_4 group. The expression of

caspase-3 and caspase-9, and the pro-apoptotic Bax were not significantly decreased. However, the anti-apoptotic Bcl2 and the ratio of Bax/Bcl2 expression were significantly different in the CS-CH_4 group, indicating the relative dominance of anti-apoptotic pathways.

Oxidative stress markers described by biochemical assays

XOR is a key enzyme in reperfusion-induced ROS production; in addition, it can catalyse the reduction of nitrates and nitrites to NO. XOR activity and tissue NOx levels were both significantly decreased when CS-CH_4 was applied during the cold ischemia period relative to the data for the CS group. While tissue MPO was significantly increased as compared to that of the control group, MPO activity was significantly reduced when CS-CH_4 was applied. The GSH/GSSG ratio was significantly decreased in the CS group; however, preservation of grafts in CS-CH_4 resulted in a sustained GSH/GSSG ratio.

Histology and immunohistochemistry

H&E staining showed only a mild disorganisation of the myofibrils with loss of striations and a combination of waviness, contraction bands and disruption of plasma membranes of myocytes in the CS group as compared to the controls. The architecture of cardiac myocytes was nearly normal in the CS-CH_4 storage group. These changes were not significantly different from those in the CS group, thus indicating nearly equal potential for tissue protection. The number of SERCA1 immunoreactive cardiac myocytes increased significantly in sections from CS-stored grafts as compared to the controls. In contrast, the number of immunoreactive cells was significantly reduced in the CS-CH_4 group.

***In vitro* effects of CH_4 during anoxic stress and evaluation of mitochondrial membrane potential changes**

Changes to mitochondrial membrane potential have been characterized by means of the potential-sensitive fluorophore safranin. Substrates of respiratory complexes induced a significant hyperpolarization in the mitochondrial membrane under normoxic conditions. In contrast, hyperpolarization was eliminated in the A/R group. Substrate-induced changes in membrane potential were partially preserved by CH_4 supplementation. CH_4 applied during the anoxic period lowered the amount of H_2O_2 production in leak states. In terms of oxygen consumption, we investigated complex I and complex II-linked respiration separately. CH_4 significantly decreased the oxygen consumption of complex I, while it had no effect on complex II-linked respiration under normoxic conditions. In contrast, CH_4 treatment in the A/R+CH_4 group significantly improved the oxygen consumption of complex II compared to complex I.

Extramitochondrial Ca^{2+} -induced membrane potential changes

In response to anoxia the extent of hyperpolarization was less prominent compared to normoxic group. This was most probably due to the non-selective Ca^{2+} efflux through mPTPs. However, CH_4 treatment resulted in significantly lower Ca^{2+} efflux. These results are in accordance with the membrane potential measurements where incubation of the mitochondria with 2.2% CH_4 maintained the hyperpolarization. Interestingly, CH_4 treatment in the normoxia group caused Ca^{2+} efflux to show no reactivity to substrates also seen in membrane potential measurements.

Of note, these results are preliminary because this method was originally developed to measure extramitochondrial Ca^{2+} movement on liver mitochondria. The magnitude of Ca^{2+} currents may be different in cardiac mitochondria; therefore, our method requires further validation.

DISCUSSION

Myocardium has particularly poor tolerance to prolonged ischemia, and the issue of preservation is a major concern in transplantation. CS solution is generally used in clinical practice; therefore, it is an appropriate testbed for alternative options. Several gas mediators, such as NO, carbon monoxide (CO) and hydrogen sulphide (H_2S) have already been tried as additives to solutions in transplantation models, assuming that a potential efficacy could be related to their tendency to react with biologically important molecules. In contrast, CH_4 is non-toxic *in vivo*; considered to be a simple asphyxiant, which means that hypoxia might occur when an increasing concentration of CH_4 displaces inhaled air in a restricted area and the concentration of oxygen is reduced. Nevertheless, there are pertinent data which demonstrate that CH_4 can modulate NO-, CO- and H_2S-linked reactions in living systems.

During circulatory arrest, depletion of mitochondrial substrates is a major contributor to Ca^{2+} influx-mediated membrane dysfunctions. As a result of CH_4 enrichment, the mitochondria were more responsive to ADP utilization, which contributed to the maintenance of OxPhos. Furthermore, cytochrome c release, a sign of mitochondrial inner membrane injury, was also reduced. The process of cold ischemia-induced cellular damage with the dual contribution of ER and mitochondria is relatively well characterized. Hypoxic conditions trigger changes in cytoplasmic resting potential and, through the activation of ER-mediated Ca^{2+} transport, increase the expression of Hif1α, one of the key initial factors in the cascade of events, which will finally lead to cell apoptosis or necrosis. Our results demonstrate that cold ischemia and graft storage activated all these participants, starting from higher SERCA1 protein levels reflecting an increased Ca^{2+} pump function in the ER and elevated Hif1α expression in

cardiomyocytes. In addition, higher intracellular Ca^{2+} can activate GSK-3β, the pro-apoptotic factor in the intrinsic mitochondrial apoptotic pathway. More importantly, if the preservation solution was supplemented with CH_4, the expression of individual genes in the proposed signaling pathway was also reduced.

The role of complex I and complex II in the post-ischemic cardiac mitochondrial respiration was addressed in more detail. As a result of CH_4 treatment, respiration was inhibited when glutamate + malate was used as a complex I substrate but not with Succ as a complex II substrate. This finding suggested that the addition of CH_4 resulted in a decreased electron flux through complex I but did not alter the Succ oxidation through complex II. Based on these findings, the drop in net ROS production from mitochondria with preserved Succ oxidation in the presence of CH_4 is most likely directly related to the inhibition of complex I. However, CH_4 appears to provide a blockade of electron transport in complex I in contrast to a complete blockade of oxidative metabolism during reoxygenation.

The mechanism of protection likely included a blockade of the electron transport in complex I and decreased ROS generation. Reversible deactivation of mitochondrial complex I is an intrinsic mechanism, which provides a fast response of the mitochondrial respiratory chain to oxygen deprivation. However, subsequent re-oxygenation leads to ROS generation due to the rapid burst of respiration. Under normoxic conditions, a high level of NADH can drive forward electron flow with superoxide generation at the flavin mononucleotide moiety located near the NADH binding subunit. During re-oxygenation, reverse electron flow driven by a reduced ubiquinone (ubiquinol) pool and high proton motive force can generate ROS when electrons flow back from ubiquinol to Complex I. CH_4 treatment restricts the forward electron transfer within complex I in control mitochondria while effectively inhibiting RET in post-ischemic mitochondria.

Mitochondrial complex I has two conformations with different catalytic activities: an active state (A) and a deactivated state (D), which are present in A/D equilibrium in a ratio of 9:7 under physiological conditions. The A/D transition occurs during ischemia/anoxia, as an intrinsic mechanism which produces a rapid response of the mitochondrial ETC to oxygen deprivation. Modulating factors of the A/D transition include the availability of oxygen, the reduced NAD pool in the matrix, the temperature and the pH. The physiological role of the accumulation of the D-form in anoxia is most probably to protect mitochondria from ROS generation due to the rapid burst of respiration following re-oxygenation. The transient preservation of complex I in the D-form was implemented as a successful strategy against reperfusion injury in the post-ischemic brain and heart. Although the entire NAD pool is

reduced under ischemic/anoxic conditions, the nucleotide at the flavin site could be rapidly reduced by the RET directly from ubiquinol at the beginning of re-oxygenation.

SUMMARY OF NEW FINDINGS

- Our study has demonstrated that CH_4 enrichment of preservation solution during cold ischemia results in increased graft protection in isogenic rat heart transplantation.

- The CH_4-containing CS effectively reduces the degree of oxido-reductive stress in myocardial samples as evolves by maintained GSH/GSSG ratio and lower NOx level in the reperfused myocardium together with decreased tissue MPO and XOR activity.

- The underlying mechanism is related to the effect of CH_4 on ER stress and the structural and functional integrity of cardiac mitochondria.

- The interaction between the mitochondria and ER involves the changes in extramitochondrial Ca^{2+} fluxes.

- The Ca^{2+}-induced mitochondrial membrane potential changes are decreased by anoxia, however, CH_4 can restore the magnitude of hyperpolarisation.

- The anti-oxidative effects of CH_4 are related to the reduced mitochondrial ROS generation via a partial blockade of electron transport in complex I.