The role of mitochondria in reactive nitrogen species production and in restoring energy levels of oxidatively injured cells

Ph.D. Thesis

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1. Introduction

Cell therapies of the heart after myocardial infarction or ischemic heart disease are aimed to assist regenerative processes to reduce infarct size and to improve cardiac function. Hypothesises regarding the mechanisms behind the beneficial effects of cell transplantation include: cell transdifferentiation, cell fusion or the presence of direct cell-to-cell contacts between host and graft cells. A possible role for mitochondria in the regeneration process arose when the ability of mitochondria to move between cells was determined about a decade ago. Applying cell therapies for the regeneration of the heart still faces many questions, such as choosing the ideal cell type, optimizing timing of transplantation and characterizing the molecular mechanism behind the regeneration process.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) formation increases in ischemia. Mitochondria are the major site of ROS and RNS production and also the target of free radicals in the cells. Nitric oxide (NO) is involved in several signaling pathways related to a diverse array of cell functions. In the myocardium, NO is produced by the enzyme nitric oxide synthase (NOS), which is present in three isoforms: neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). Several studies raised the possibility that a distinct mitochondrial NO synthase enzyme (mtNOS) exists, although this theory was challenged recently and skepticism regarding its existence, origin, as well as functional role has remained.

Excess production of ROS results in the overactivation of the nuclear enzyme poly (ADP-ribose) polymerase (PARP), which leads to mitochondrial dysfunction and cell death. PARP, residing in the nucleus, builds up poly (ADP-ribose) polymers on different proteins, such as histones, and on PARP itself, regulating enzyme activity. Some studies described (ADP-ribosyl)ation of proteins in mitochondria either via an enzymatic pathway or via the non-enzymatic transfer of nicotinamid adenine dinucleotide (NAD^+) -

glycohydrolase liberated ADP-ribose onto acceptor proteins. In spite of the presence of different (ADP-ribosyl) transferases and free ADP-ribose moieties in mitochondria, very limited information is available on the acceptor proteins, as well as on the nature of the enzymes catalyzing intra-mitochondrial poly (ADP-ribosyl)ation (PARylation) reactions. Furthermore, the contribution of oxidative and nitrosative stress to mitochondrial poly (ADP-ribose) (PAR) formation is not yet understood. The existence of a genuine PARP enzyme in the mitochondrial matrix is still subject to debate.

2. Aims

The main objective of the present thesis was to elucidate the role of mitochondria in oxidative and nitrosative stress and in the regeneration of cardiac muscle after ischemic injury.

Our specific questions were:

2.1. Can healthy cells rescue their oxidatively injured cardiomyoblast neighbors from cell death? What is the role of mitochondria in the rescue process?

2.2. What kind of proteomic changes occur in the mitochondria following oxidative and nitrosative stress? Which enzyme might be responsible for the poly (ADP-ribosyl)ation of proteins in the mitochondria?

2.3. Does mitochondrial nitric oxide synthase exist? What are the other possible sources of reactive nitrogen species in the mitochondria?

3. Materials and methods

3.1. In vitro ischemia model

H9c2 cardiomyoblasts were cultured in high glucose DMEM, and then underwent oxygen and glucose deprivation (OGD) for 2.5 hours. Cells were

labeled with the fluorescent dye Vybrant DiO (1:200, 30 minutes, 37 ^{0}C). After OGD, Vybrant DiD-labeled various cell types were added to the culture. These cell types were as follows: healthy H9c2 cells, mitochondria-damaged H9c2 cells, healthy mesenchymal stem cells (MSCs), healthy human embryonic kidney cells (HEKs) or healthy Madin-Darby canine kidney cells (MDCKs). Mitochondrial dysfunction was achieved with ethidium bromide (EtBr, 50ng/ml in the cell culture media for 2 months) or F16 treatment (48 h, 200μM in the cell culture media). The Vybrant DiD-labeled cells were co-cultured with the OGD treated, Vybrant DiO-labeled H9c2 cells for 24 h. To follow the movement of mitochondria, all cells were stained with MitoTracker Red in a dilution of 1:2000 for 10 minutes at 37°C. Cells were seeded on glass coverslips and their movement and the formation of tunneling membrane tubes was observed overnight using a Zeiss LSM 510 META confocal microscope. Pictures were taken every 15 minutes at high resolution, with a 63x objective. Cells were grown in 12-well plates for calculating the live cell number after EtBr and F16 treatment. Seeded cell number was always 15 000 in each well. 2x2 tile scan pictures were taken from two different locations from each well. Using the ImageJ cell counter software dead cardiomyocytes, live cardiomyocytes and healthy rescue cells were labeled with different colors and counted. The number of cells is expressed as mean ± SEM, * $p< 0.05$ with ANOVA analysis.

Green fluorescent protein (GFP) and red fluorescent protein (RFP) labeling was carried out using cells, which were transfected with plasmid DNA coding 2MTS-EGFP or 2MTS-mRFP. These constructs use the N-terminal target sequence of the human cytochrome c oxidase subunit 8A in duplicate fused to the N terminus of the fluorescent proteins. $3x10^6$ cells were transfected with 4 μg of DNA in 100 μl Neon tips, using the following conditions: 1150 V, 30 ms, 2 pulses.

3.2. Proteomic analysis experiments

In search or PARP-like enzymes in the mitochondria and poly (ADP ribosyl)ated proteins, we isolated mitochondria from rat liver either using the discontinuous Percoll gradient method or using ice-cold sucrose buffer (0.25M sucrose, 0.5mM EDTA, 5mM TRIS, pH 7.2). Fresh, respiring mitochondria were treated with H_2O_2, S-nitroso glutathione (GSNO) or S-nitroso-N-acetylpenicillamine (SNAP) to induce oxidative and nitrosative stress (each compound was 3 mM, for 30 minutes on ice). Each group received +/- 5mM 5-methoxyindole-2-carboxylic acid (MICA), a specific inhibitor of dihydrolipoamide dehydrogenase, as pretreatment for 30 minutes. Proteins were isolated from these mitochondrial preparations and analyzed with Western blot. 10μg protein was loaded from each sample onto 4-12% Bis-Tris Invitrogen mini gels. Proteins were transferred to Invitrogen nitrocellulose membranes. Membranes were blocked in 10% non-fat dry milk in TBS containing 0.05% Tween for 1 hour at room temperature, and then washed 3 times for 5 minutes in TBS-Tween. Membranes were incubated with anti-PAR rabbit polyclonal primary antibody in the dilution of 1: 5000 (Calbiochem) or anti-PARP rabbit polyclonal primary antibody in the dilution of 1: 4000 (Calbiochem) overnight at 4^0C, followed by 3 times 5 minutes washing steps, then incubation with anti-rabbit IgG-HRP secondary antibody in the dilution of 1: 2000 (Cell Signaling) for 1 hour at room temperature. After washing the membrane 4 times 5 minutes in TBS-Tween, Amersham ECL detection kit was used to visualize protein bands. Two-dimensional gel electrophoresis was also carried out in the control and H_2O_2 treated groups for better separation of the proteins. Four PAR-positive protein bands from the H_2O_2 treated group; and one PAR-positive protein band from the NO-donor treated group were isolated from the silver stained gels and analyzed with mass spectrometry. The PARP-like enzymatic activity of one of the identified proteins – namely dihydrolipoamide dehydrogenase – was tested via two different enzyme activity assays. One-way ANOVA followed by

Tukey's multiple comparison test was carried out in the enzyme activity assay experiments. Six parallel experiments were run in each group. Optical density results are shown as mean ± SEM (*: $p < 0.05$).

3.3. Nitric oxide measurement experiments

Mitochondria were isolated from the liver, heart and brain of C57/BL6 mouse. In one set of experiments, to find the source of NO production in mitochondria, samples were treated with the NOS inhibitors: L-nitroarginine-methyl-esther (L-NAME, 1mM, or 10 mg/ml for 3 days in the drinking water of the animals) or L-N-methylarginine (L-NMMA, 1 mM). NO and RNS production was measured by flow cytometry using diaminofluorescein (DAF–FM, 7 μM). Forward-scatter, side-scatter and green fluorescence (FL1-H) were recorded by a BD-FacsCalibur flow cytometer from 100,000 events in each preparation. Data were evaluated by the CellQuestPro software. To check whether the presence of $ONOO^-$ also increases DAF-FM signals, mitochondria samples received $ONOO^-$ scavengers: tetrakis 2- triethylene glycol monomethyl ether (pyridil porphyrin (2-T(PEG3)- PyP) (FP15, 100 μM), or 5,10,15,20-tetrakis (4-sulfonatophenyl) porphyrinato iron (III) chloride (FeTPPS, 100 μM). To rule out the role of superoxide and H_2O_2 in the contribution to DAF-FM fluorescence a superoxide dismutase mimetic (M40401) and catalase were also applied.

In another set of experiments we measured the NO and RNS production of the respiratory chain complexes. The following treatments and their combinations were used to test the involvement of the mitochondrial respiratory chain: the complex I blocker rotenone (10 μM) in the absence of mitochondrial substrates malate and glutamate or in the presence of K-succinate (5mM); the succinate dehydrogenase (complex II) inhibitor 3-nitropropionic acid (3-NPA, 100 μM); the complex III inhibitor myxothiazole (10 μM), the complex IV inhibitor KCN (100 μM) and the protonophore carbonyl cyanide 4 (trifluoromethoxy) phenylhydrazone (FCCP, 10 μM). The rate of ROS–RNS generation in heart

mitochondria was detected in the presence of 250 μM of 1-hydroxy-3-carboxypyrrolidine (CPH). Mitochondria were incubated for 20 min at 22 ± 1.5 ^{0}C, then the samples were placed in glass capillars and electron paramagnetic resonance (EPR) spectra were recorded with an X-band MiniScope 200 EPR spectrometer. Upon reaction with ROS or RNS CPH is transformed into a stable CPz radical (3-carboxy-proxyl). Calibration data were obtained in mitochondria-free buffers containing increasing concentrations of the probe 3-CP. Nitric oxide release was measured in the head-space gas of the mitochondria preparations. Mitochondria were suspended in 300 μl of buffer containing glutamate and malate (5 mM each) and incubated for 45 min in 1500 μl eppendorf tubes. The NO content in the headspace gas was measured by a chemiluminescent reaction (Exhaled NO Analyzer). Sodium nitroprusside solution (SNP, 10^{-7}–10^{-3} M) was used as a positive control.

4. Results

4.1. The role of mitochondria in rescuing postischemic cardiomyoblasts

The majority of the cells died or suffered major structural changes during OGD. Healthy H9c2 cells were co-cultured with the OGD treated H9c2 cells for 24 hours. Cells attached to the surface of the culture dish and made contacts with their healthy and also with their OGD treated neighbors. Healthy H9c2 cells were able to significantly increase the surviving cell number in the co-culture.

In order to investigate the role of mitochondria in this ‘rescue’ mechanism not only healthy H9c2 cells were added to the OGD treated cells. In a set of experiments the additional H9c2 cells were pretreated with 50ng/ml EtBr in the cell culture media for 2 months. This compound affects only mitochondrial DNA at this concentration and in this time frame. Healthy H9c2 cells were able to significantly improve the survival of their OGD treated neighbors, on the contrary, addition of EtBr treated, mitochondria-damaged H9c2 cells did not increase the number of the surviving cells (Figure 1.). The statistical analysis

shows the number of live cells as mean ± SEM and * $p < 0.05$. The pretreatment of the additional cells with F16, another mitochondrial toxin also prevented this rescue effect.

To confirm the role of mitochondria in saving oxidatively damaged cardiomyoblasts high resolution time lapse images were taken overnight with a confocal microscope. We detected the movement of cells towards each other and the formation of membrane bridges between OGD treated and healthy cells. Healthy cells also formed membrane tubes among themselves, and similarly, we were able to detect membrane bridges between OGD treated cells. We labeled the mitochondria of the cells with MitoTracker, and we were able to follow mitochondrial movement. Our results show that mitochondria-like particles are drifting through these membrane bridges between neighboring cells (Figure 1.). We applied another approach to track mitochondrial movement by the transfection of H9c2 cells with green fluorescent and red fluorescent proteins with a mitochondrial target sequence. One cell population was labeled with GFP and subjected to OGD, then a healthy cell population labeled with RFP was added as rescue cells. Although in this experiment the mitochondria in the two cell populations were easily distinguishable, we did not observe the entry of mitochondria into another cell, neither found double-labeled cells in the co-culture. Sporadic yellow colored mitochondria were seen in areas of high confluency, however, the 3 dimensional images constructed from the Z-stack scans could not exactly locate the GFP-labeled mitochondria inside the RFP-labeled cells indicating that the rare occurrence of co-localization is possibly due to overlap rather than mitochondrial transfer.

In search for easily accessible and more suitable cells in the regeneration process, we screened cell types at various stages of differentiation. OGD treated H9c2 cells were co-cultured with healthy mesenchymal stem cells, healthy H9c2 cells, with human embryonic kidney cells, or with adult Madin-Darby canine kidney cells. Our results show that the addition of MSCs and healthy H9c2 cells

to the OGD treated group can significantly improve the cell survival ratio. Human embryonic kidney cells also helped the survival of OGD treated H9c2 cells, but adult canine kidney cells were not able to improve the cell survival ratio in this experiment.

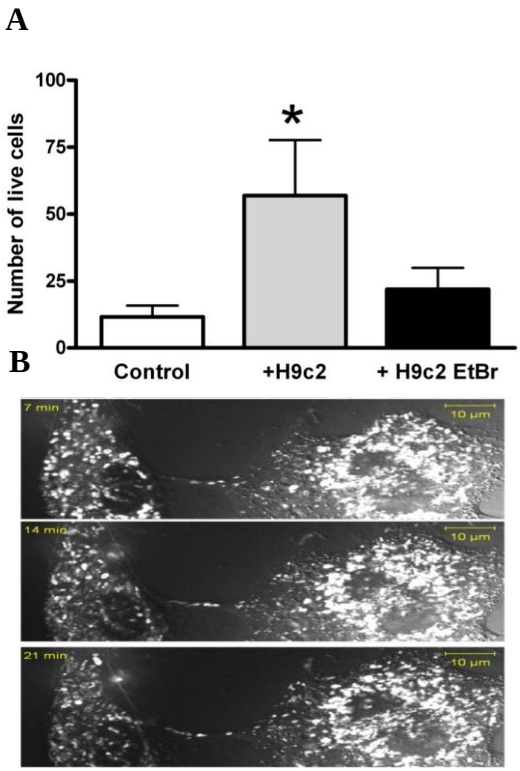

Figure 1. Mitochondria play a role in saving oxidatively injured cardiomyoblasts. The addition and co-cultivation of healthy H9c2 cells with the OGD treated cells significantly increased the live cell number. EtBr pretreatment of the additional H9c2 cells resulted in altered mitochondrial function, and these EtBr treated H9c2 cells were not able to significantly increase the live cell number in the co-culture. OGD treated cells and healthy cardiomyoblasts develop connecting membrane bridges. MitoTracker labeled mitochondria are traveling through these tunneling membrane tubes from one cell to another.

4.2. Proteomic changes in mitochondria in oxidative and nitrosative stress

Treatment of rat liver mitochondria with H_2O_2 and with the NO-donor GSNO resulted in extensive poly (ADP-ribosyl)ation of the proteins. 2D gel electrophoresis coupled with Western blot analysis and mass spectrometry identified 5 PARylated proteins: dihydrolipoamide dehydrogenase, mitochondrial aldehyde dehydrogenase, ATPase, ornithine transcarbamylase and carbamoyl-phosphate synthase (Figure 2.).

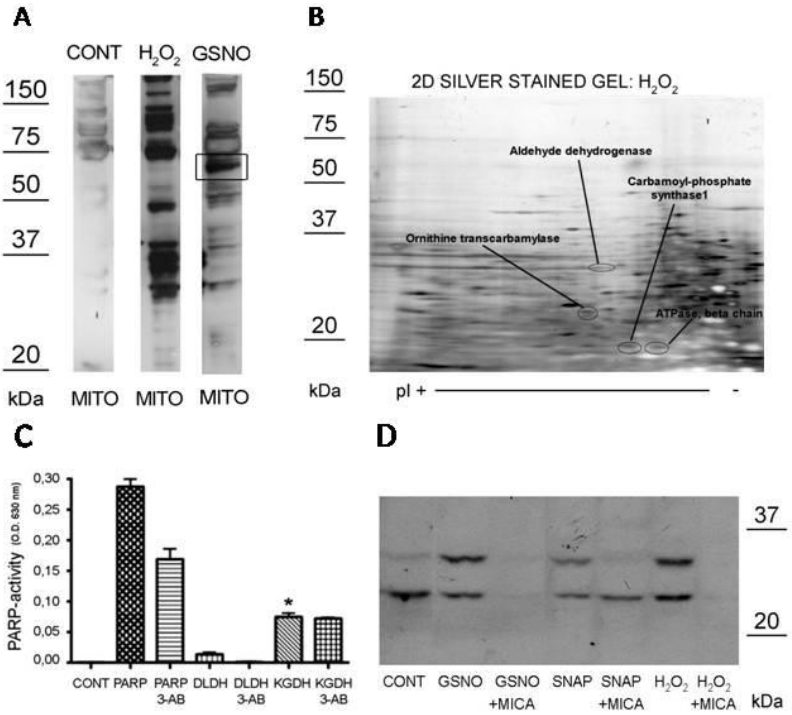

Figure 2. Proteomic analyses of mitochondrial enzymes following oxidative and nitrosative stress. H_2O_2 and GSNO treatment results in extensive PARylation of mitochondrial proteins (panel A). The identified PARylated proteins are important members of the respiratory chain, Krebs cycle or the urea cycle (panel B). DLDH did not have a significant PARP-like activity, but KGDH showed significant PARP-like enzymatic activity, which was not inhibitable by the PARP inhibitor 3-AB (panel C). The extra PAR-positive bands caused by GSNO, SNAP or H_2O_2 treatment were diminished by MICA, the specific inhibitor of DLDH (panel D).

DLDH uses the same substrate as PARP, and shows strong PARylation in response to nitrosative agents just like PARP, thus we hypothesized that DLDH may have an intrinsic PARP-like enzymatic activity. DLDH serves as the E3 component of pyruvate dehydrogenase, alpha-ketoglutarate dehydrogenase (KGDH) and glycine decarboxylase complexes in the mitochondria. Using a colorimetric PARP enzyme activity assay kit, we were not able to detect

significant PARP activity of DLDH compared to the 1U PARP positive control. However, KGDH showed significant PARP-like enzyme activity. The prototypical PARP inhibitor 3-aminobenzamide inhibited PARP activity but did not affect the activity of KGDH (Figure 2.). Incubation of the mitochondrial samples with H_2O_2 or different NO donors, such as GSNO and SNAP resulted in extra PAR-positive bands on Western blot. MICA, the specific inhibitor of DLDH diminished these extra PAR-positive bands (Figure 2.).

4.3. Sources of reactive nitrogen species production in mitochondria

Isolated respiring mitochondria showed a similarly strong DAF fluorescence regardless of the presence or absence of the NOS substrate L-arginine or modulation of Ca^{2+} levels, the obligatory cofactor of NOS. Blockade of a potentially mitochondrial localized NOS by the competitive inhibitors L-NAME and L-NMMA failed to reduce mitochondrial DAF fluorescence. Prolonged in vivo application of L-NAME and supplementation of the mitochondria isolation buffers with L-NAME also failed to inhibit DAF fluorescence, making it very unlikely that the hypothetical mtNOS is responsible for the RNS production. Addition of $ONOO^-$ decomposition catalysts FeTPPS or FP15 to the mitochondria markedly decreased the fluorescence. Application of a SOD mimetic (M40401) or catalase or the co-application of both failed to affect the fluorescent signal, indicating that the DAF-FM fluorescence in mitochondria is not due to superoxide or hydrogen-peroxide but rather dependent on a nitrogen species such as $ONOO^-$. These experiments confirmed that mitochondria produce RNS independently from the L-arginine– NOS pathway or reactive oxygen species. Mitochondrial RNS production was also evaluated by the application of the spin trap CPH followed by electron spin resonance measurements. Respiring mitochondria produced a significant amount of reactive species that was not sensitive to NO synthase inhibitors, complementing the data gathered by DAF fluorescence. Since the presence of

SOD mimetics or $ONOO^-$ decomposition catalysts in the reaction mixture interferes with the ESR measurements, the following experiments were carried out by flow cytometric quantitation of DAF fluorescence. Mitochondrial DAF fluorescence was markedly inhibited when the organelles were denatured by three cycles of freezing and thawing or heating the preparations to 95^0C. Similarly, DAF fluorescence was reduced when mitochondrial respiration was blocked by the application of rotenone or when respiratory substrates malate and glutamate were not supplemented in the medium. Inhibition of complex II in the presence of complex I substrates, or mitochondria respiring on succinate in the presence of the complex I inhibitor rotenone had similar fluorescence values, indicating that either complex I or complex II can supply electrons for the reaction. Inhibition of the ubiquinone oxidation site of complex III slightly increased the fluorescence, supporting that the ubiquinone pool is necessary for the reaction. Inhibition of complex IV or collapsing the proton gradient by FCCP had no effect on RNS production. We attempted to locate a NOS-like protein in mouse heart mitochondria by Western blotting with a set of anti-NOS antibodies. All of the antibodies reacted well with the positive controls but no specific mitochondrial bands were found at the expected molecular weights (144 kDa for eNOS, 150–160 kDa for nNOS and 125 kDa for iNOS). In addition, we confirmed these results in human tissues and did not find any NOS positive protein band in the mitochondria samples at the expected molecular weights (Figure 3.). We attempted to measure mitochondrial NO release by the gas-phase chemiluminescent technique, which is a direct and very sensitive method for the quantitation of NO in gas phase and it is routinely used in clinical settings to quantify the NO content of exhaled air. We detected as low as a few ppb NO in the headspace air of an NO donor, but in case of mitochondria the NO content was below the detection limit (Figure 3.). Even though we could not detect mtNOS in mitochondria samples by Western blot, these results do not exclude the possibility that low levels of NO or other reactive nitrogen species

are produced and metabolized in the mitochondrion without leaving the organelle.

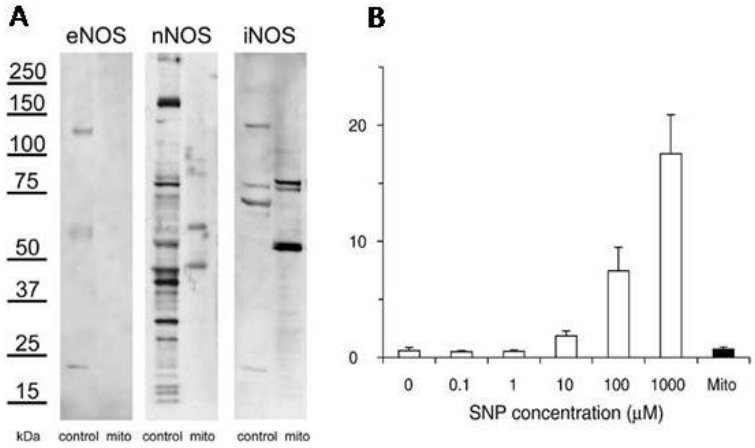

Figure 3. NOS isoforms and NO release in human heart mitochondria. Panel A shows Western blots with anti-NOS antibodies. Note that the positive controls contain a respective 140–160 kDa band and several unspecific lower molecular weight bands, while the mitochondria do not have a specific signal. Panel B shows gas-phase NO measurements where the mitochondria samples (n=6) did not produce significant amount of NO compared to the sodium nitroprusside (SNP) positive control.

5. Discussion

5.1. Although heart is one of the least regenerative organs, replacement of injured cardiac tissue gained great attention in the last decade, as heart diseases are the leading cause of hospitalization, morbidity and mortality of man in the Western world. No agreement has been reached among scientific groups on the type of the most appropriate donor cells, on the techniques of cell application, and on the selection of the appropriate group of patients for cell therapy. Despite these controversies there is a general agreement that cell transplantation therapies of the injured heart reduce scar size and improve heart function. Our earlier studies showed that MSCs can improve the survival of ischemic

cardiomyoblasts in co-culture. In this study we focused on the beneficial effects of healthy H9c2 cells on the survival of oxidatively damaged H9c2 cells in an *in vitro* ischemia model, and found that healthy H9c2 cells are able to increase the cell survival to the same extent as mesenchymal stem cells. Cells at various differentiation states had different effect on the cell survival ratio after oxygen-glucose deprivation. Up to date, many different hypotheses - such as transdifferentiation, cell fusion and direct cell-to-cell interactions - arose on the mode of action how neighboring cells can help each other to survive upon oxidative stress. In our present study we found tunneling membrane tube formation between oxidatively damaged and healthy cardiomyoblasts, and we were also able to follow mitochondrial movement in these membrane bridges between cardiomyoblasts. We found that healthy cardiomyoblasts can save their oxidatively damaged neighbors from cell death in co-culture, but at the same time EtBr or F16 treated mitochondria–damaged cardiomyoblasts were not able to save their neighbors from ischemic injury. We think that healthy mitochondria are necessary to achieve this 'rescue' effect and also showed that the presence of tunneling membrane bridges between cells enables mitochondrial traffic between OGD treated and healthy cardiomyoblasts in cell culture. Although regenerating the heart is in the focus of research for the last ten years, it should be taken into consideration that there are still many gaps in our current knowledge. Cell-based cardiac therapies have the potential to improve heart function, but further research and long-term follow-up studies will be needed before safe clinical implications.

5.2. As we found an important role for mitochondria in our *in vitro* oxidative injury experiments, we were trying to find out what kind of molecular changes can occur in mitochondria following oxidative stress. Oxidative and nitrosative injury leads to the post-translational modification of several proteins, including mitochondrial enzymes involved in respiration or energy metabolism. Poly (ADP-ribosyl)ation is a post-translational modification that occurs in the

nucleus under normal conditions; however, overactivation of the nuclear PARP enzyme in hypoxia results in excess poly (ADP-ribose) polymer formation. PAR polymers were suggested as the moderator in the nuclear – to – mitochondrial crosstalk, transferring 'commit-suicide' signals and encouraging mitochondria to release AIF and cytochrome- c. How exactly PAR polymers communicate with mitochondria is not clear, but experimental evidence showed that poly (ADP-ribosyl)ation is not the privilege of the PARP enzyme, and is not restricted to the nucleus. Some studies found PARP activity and poly (ADP-ribosyl)ation in the cytoplasm and also in the mitochondria. In this study we show that oxidative and nitrosative stress leads to the PARylation of several mitochondrial proteins, including the members of the Krebs cycle, urea cycle and the respiratory chain. ATPase and mtALDH are inhibited in oxidative stress, Otc has decreased level and CPS-1 releases from the mitochondria following oxidative or nitrosative stress. Inhibition of ATPase leads to decreased ATP levels and finally to mitochondrial dysfunction. CPS-1 initiates the urea cycle and deficiency or decreased activity of the enzyme leads to hyperammonemia. Otc also has a role in the urea cycle and in arginine metabolism. In oxidative stress Otc preprotein does not translocate from the cytoplasm to the mitochondria causing decreased level of the mature protein. Oxidative stress results in lipid peroxidation and in the formation of toxic aldehydes such as, 4-hidroxy-2-nonenal (HNE), malondialdehyde (MDA). These toxic aldehydes inhibit mtALDH and also inhibit the mitochondrial respiration at complex II. and III. leading to changes in the mitochondrial transmembrane potential (Figure 4.).

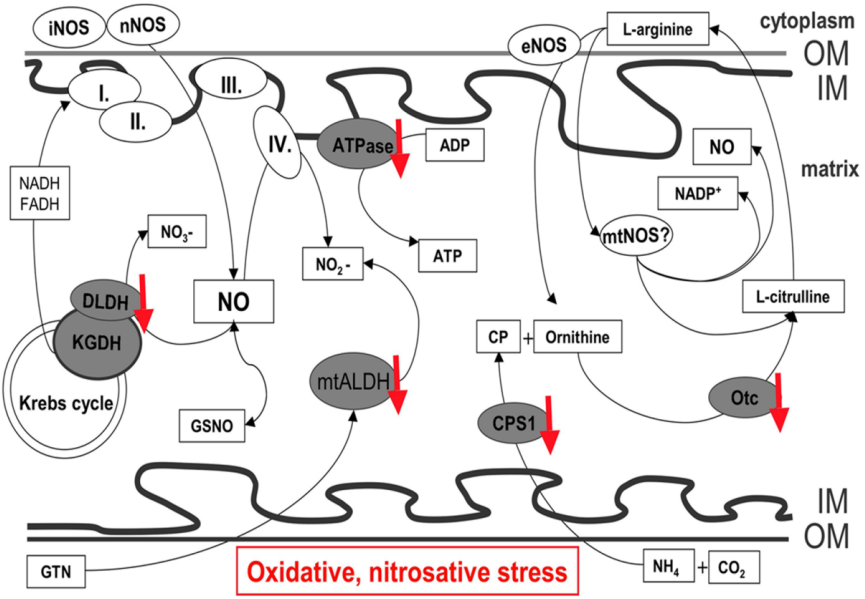

Figure 4. Mitochondrial proteomic changes following oxidative and nitrosative stress induced by H_2O_2 or NO-donors. The identified proteins are all important players in mitochondrial energy maintenance. Oxidative stress and nitrosative stress leads ti the inhibition or decreased activity of these enzymes. OM: outer membrane, IM: inner membrane, I., II., III., IV. : Respiratory chain complexes.

DLDH, being a part of the PDH and KGDH enzymes plays an essential role in cell energy maintenance. DLDH has many different activities, which are influenced by the presence of free radicals, or by pH changes that occur in oxidative stress. In our experiments we showed by mass spectrometry that DLDH is PARylated in rat liver mitochondria following GSNO treatment. The application of MICA – a specific DLDH inhibitor – have diminished or reduced the number of PAR-positive bands in isolated mitochondria samples following oxidative and nitrosative stress. In vitro enzyme activity assays showed PARP-like activity of KGDH in Western blot and spectrophotometric analysis. DLDH changes its oligomeric state under mitochondrial acidification in oxidative stress

and has decreased activity. The inhibition of KGDH in oxidative stress is crucial for maintaining the energetic state of the cell. KGDH is mainly inhibited by H_2O_2, but HNE, superoxide, peroxynitrite, and NO itself also decreases the activity of the enzyme. PARylation of DLDH may be one of the factors responsible for the decreased activity of the KGDH enzyme in oxidative and nitrosative stress. Further studies are needed to determine whether KGDH is a physiological regulator of mitochondrial NAD and PAR metabolism, and whether it contributes to the pathological alterations in poly (ADP-ribosyl)ation in various diseases.

5.3. PARP is not the only enzyme that might have a mitochondrial counterpart. As it was mentioned earlier a putative mitochondrial nitric oxide synthase is believed to exist and believed to produce NO in the mitochondria. NO production in mitochondria was observed by several laboratories using diaminofluorescein fluorescent probes. However, in spite the strong DAF fluorescence signals in mitochondria, identification of any NOS-like protein in the mitochondria faces difficulties. Using several different antibodies against the known NOS isoforms we were not able to detect NOS-positive protein bands in human heart mitochondria samples; nor were we able to detect significant amounts of NO from the headspace air of mitochondria samples. DAF was also shown to be sensitive to peroxynitrite that is readily formed from superoxide and NO in the mitochondria. In this study we tried to investigate if NO is produced by mtNOS in the arginine-to-citrulline conversion pathway in the mitochondria. Here we show that mitochondrial DAF fluorescence cannot be reduced even by rather high concentrations or prolonged application of NOS blockers. Withdrawal of L-arginine and Ca^{2+}, and the co-application of catalase and a SOD mimetic also failed to decrease DAF fluorescence in mitochondrial preparations, further supporting the idea that no arginine-dependent NOS is involved in the reaction. In contrast, the application of the $ONOO^-$ decomposition catalysts FP15 and FeTPPS, or denaturating the mitochondria, as

well as the inhibition of mitochondrial respiration in the lack of substrates successfully prevented DAF fluorescence. Summarizing the experiments with respiratory chain inhibitors it is clear that electron transport through complexes I and II to the ubiquinone cycle is necessary for RNS production, however, the lack of a proton gradient or ATP production does not affect it. NO produced in different compartments of a cell or even in adjacent cells can easily diffuse into the mitochondria, finding superoxide readily there to form peroxynitrite that remains the main candidate responsible for the DAF fluorescence signals.

Controversies regarding the NO production by a putative mtNOS emerge mainly from the difficulties of reproducing experiments from various laboratories, because of the different mitochondria isolation protocols, contamination of the mitochondria samples with other cellular components, such as the known eNOS isoform that was shown to be attached to the outer mitochondrial membrane. The eNOS enzyme is anchored to juxta-mitochondrial smooth endoplasmic reticulum and a pentabasic amino acid sequence was identified in the autoinhibitory domain of eNOS, which is responsible for the mitochondrial docking of the enzyme. These findings indicate that mtNOS may indeed be a cellular NOS enzyme, which is loosely attached to the outer surface of mitochondria. Although there is not enough experimental evidence to prove it, one can hypothesize that mitochondrial attachment plays a role in the regulation of NOS activity, and thus docking of an active NOS on the outer membrane, with resultant NO production, regulates respiration. Genomic approaches were not able to detect a mitochondrial NOS gene in the genome, thus the putative NOS protein should be transported from the cytoplasm to the mitochondrial matrix via active transport. However, the known NOS isoforms do not possess the obligatory mitochondrial target sequence tag, required for the transportation of the protein to the mitochondria. In addition, the whole human heart mitochondrial proteome was described, but not any NOS isoforms were found among the mitochondrial proteins.

6. Conclusions

6.1. Healthy cardiomyoblasts and mesenchymal stem cells can save their postischemic neighbors from cell death. Mitochondria play an important role in this rescue process either by restoring energy levels in the dying cells or by inhibiting cell death signals of damaged mitochondria.

6.2. Oxidative and nitrosative stress results in extensive poly (ADP-ribosyl)ation of mitochondrial proteins, including members of the Krebs cycle, respiratory chain and urea cycle. PARylation of these enzymes might lead to their dysfunction, causing the imbalance of mitochondrial energy production and eventually cell death. Dihydrolipoamide dehydrogenase is a potential candidate, responsible for the poly (ADP-ribosyl)ating enzyme activity in mitochondria.

6.3. Mitochondria produce reactive nitrogen species independently from the L-arginine– NOS pathway. Nitric oxide synthase protein was not present neither in mouse nor in human mitochondria.

7. Publications

7.1. Publications closely related to the thesis

1. Cselenyak A, **Pankotai E**, Horvath EM, Kiss L, Lacza Z. Mesenchymal stem cells rescue cardiomyoblasts from cell death in an in vitro ischemia model via direct cell-to-cell connections. BMC Cell Biol. 2010 Apr 20;11(1):29. **IF:3,16**

2. **Pankotai E**, Lacza Z, Murányi M, Szabó C. Intra-mitochondrial poly(ADP-ribosyl)ation: potential role for alpha-ketoglutarate dehydrogenase. Mitochondrion. 2009 Apr;9(2):159-64. **IF:4,262**

3. Csordás A, **Pankotai E**, Snipes JA, Cselenyák A, Sárszegi Z, Cziráki A, Gaszner B, Papp L, Benko R, Kiss L, Kovács E, Kollai M, Szabó C, Busija DW, Lacza Z. Human heart mitochondria do not produce physiologically relevant quantities of nitric oxide. Life Sci. 2007 Jan 23;80(7):633-7. **IF: 2,389**

4. Lacza Z, Kozlov AV, **Pankotai E**, Csordás A, Wolf G, Redl H, Kollai M, Szabó C, Busija DW, Horn TF. Mitochondria produce reactive nitrogen species via an arginine-independent pathway. Free Radic Res. 2006 Apr;40(4):369-78**. IF: 5,44**

5. Lacza Z, **Pankotai E**, Busija DW. Mitochondrial nitric oxide synthase: current concepts and controversies. Front Biosci. 2009 Jan 1;14:4436-43. Review. PubMed PMID: 19273361.**IF: 3,308**

7.2. Other publications

1. Cao T, Racz P, Szauter KM, Groma G, Nakamatsu GY, Fogelgren B, **Pankotai E**, He QP, Csiszar K. Mutation in Mpzl3, a novel gene encoding a predicted adhesion protein, in the rough coat (rc) mice with severe skin and hair abnormalities. J Invest Dermatol. 2007 Jun;127(6):1375-86. **IF: 4,829**

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