Investigation of the bioenergetic energy metabolism and capability of reactive oxygen species production of brain mitochondria

PhD thesis

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INTRODUCTION

Mitochondria, the powerhouse of the cell, synthesize ATP while reducing oxygen to water. This process is mediated by the electron transfer system (ETS) located in the mitochondrial inner membrane. Our body obtains energy via the catabolism of different nutrients (mainly carbohydrates and fat) leading to the generation of reducing equivalents, such as NADH and $FADH_2$. These molecules act as electron donors to the ETS and are oxidised at complex I (CI) and complex II (CII), respectively. The electrons are transferred from CI and CII, via the Q-junction, towards complex III (CIII). From there, they are then transferred to complex IV via cytochrome c to reduce oxygen into water. The redox potential of the complexes is incrementally increasing downstream the ETS allowing the transfer of electrons from donors to acceptors. This phenomenon is accompanied by the liberation of free energy, facilitating the proton translocation from the mitochondrial matrix to the intermembrane space. Because of the charge of ions, the mitochondrial matrix is negatively charged, and the intermembrane space more positively charged, resulting in an electrochemical gradient over the membrane known as the proton motive force (*pmf*) which comprises $\Delta\psi_m$ and ΔpH. This energy is used to synthesize ATP from ADP and inorganic phosphate by the ATP synthase.

The mitochondria are able to produce ATP by three different mechanisms: i) oxidative phosphorylation (OXPHOS); ii) substrate level phosphorylation (SLP) in the Krebs cycle (catalysed by succinyl-CoA ligase); and iii) adenylate kinase (AK) reaction (2 ADP $\leftrightarrow$ ATP

+ AMP). Mitochondrial SLP is attributed to succinyl–CoA ligase which is an enzyme of the Krebs cycle with two isoforms: an ATP forming isoform, succinyl-CoA + ADP + P_i ↔ succinate + CoA-SH +ATP; and a GTP forming isoform, succinyl-CoA + GDP + P_i ↔ succinate + CoA-SH +GTP. SLP can be considered as an alternative mechanism for ATP synthesis, because mitochondrial SLP is partially independent from the ETS and the mitochondrial *pmf*. Furthermore, SLP has a significant role when OXPHOS is impaired. Mitochondria have been known to both synthesise and hydrolyse ATP. The ATP hydrolytic activity of mitochondria is typically associated with the hydrolysis of glycolytic ATP and by depletion of cellular ATP. In order to hydrolyse glycolytic ATP, both the ATP synthase and ANT have to work in the reverse mode. However, the reversals of ATP synthase and ANT have been shown to not simultaneously occur because their reversal potentials are different. Also, the ATP production by matrix SLP may help to prevent the reversal of ANT and thus, could inhibit the mitochondrial utilisation of cytosolic ATP.

There is a large body of evidence suggesting that mitochondrial dysfunction is associated with several conditions such as neurodegenerative diseases, diabetes type 2, ischemia-reperfusion and cancer. In the last few decades the number of mitochondria-targeted drugs have been growing such as methylene blue (MB), which seemed to be a neuroprotective compound. The aim of our study was to investigate the effect of MB on the mitochondrial SLP in guinea-pig brain mitochondria. Due to its heterocyclic ring it can cross biological membranes; its effect is not dependent of receptor binding.

In the case of CI and CIII inhibition, NADH accumulates and inhibits in turn α-ketoglutarate dehydrogenase (α-KGDH), which catalyses the rate limiting step in the Krebs cycle. Under these circumstances, MB provides an alternative way of electron transport from NADH and $FADH_2$ to cytochrome c avoiding the inhibition of α-KGDH. The electrons are then transferred via CIV to O_2 reducing it into water and inducing ATP synthesis. Furthermore, it has been demonstrated that the MB can rescue mitochondrial membrane potential ($\Delta\psi_m$) and decrease mitochondrial calcium uptake. We investigated the effect of MB on mitochondrial SLP catalysed by succinyl-CoA in isolated brain mitochondria from guinea-pig.

Impairment of the ETS is not only accompanied with altered energy metabolism, but usually also with increased production of reactive oxygen species (ROS), which are reactive oxygen compounds produced during the metabolism of oxygen. In the cell several ROS producing organelles exist, such as mitochondria, endoplasmic reticulum, peroxisome, etc. Mitochondria however, are one of the major ROS generators where CI is a primary source of ROS and its dysfunction is relevant to several pathologies. In isolated mitochondria CI-mediated ROS generation can be triggered by: 1) NADH-linked substrates like glutamate and malate, 2) NADH-linked substrates in the presence of CI inhibitors, like rotenone, or 3) $FADH_2$-linked substrates like succinate or alpha-glycerophosphate (α-GP), which support reverse electron transfer (RET) towards CI from CII and alpha-glycerophosphate dehydrogenase (α-GPDH). RET takes

place via the Q-junction in non-phosphorylating mitochondria with high *pmf*. Therefore, RET-associated ROS production is thought to be dependent on *pmf*. It has been shown that succinate- or α-GP-fuelled RET is highly sensitive to minor changes in $\Delta\psi_m$ in mammalian and *Drosophila* isolated mitochondria. Additionally, it has been shown that a 10% decrease in $\Delta\psi_m$ (*e.g.* caused by an uncoupler) gave a 90% decrease in succinate-driven ROS production in rat heart mitochondria. It has also been demonstrated that the ΔpH appears to have a regulating effect on mitochondrial ROS (mtROS) formation. Upon acidification of the matrix, the mtROS production is decelerated, which can be explained by the stabilisation of the semiquinone radicals. According to Lambert and Brand, succinate-driven mtROS production is more dependent on ΔpH than on $\Delta\psi_m$, as detected in mitochondria isolated from rat skeletal muscle. To the contrary, Selivanov and co-workers revealed that mtROS generation is significantly affected by the actual value of pH itself (extramitochondrial pH; pH_{extra} and intramitochondrial pH; pH_{in}) and not particularly influenced by ΔpH or $\Delta\psi_m$, as measured in rat brain mitochondria.

Our aim was to clarify, using isolated brain and heart mitochondria, which of the two components of the *pmf* have the more pronounced role in the regulation of mtROS production. $\Delta\psi_m$ and ΔpH usually change in the same direction: addition of uncoupler decreases both $\Delta\psi_m$ and ΔpH. With ionophores, like valinomycin and nigericin, it is possible to dissect the two components of *pmf;* $\Delta\psi_m$ and ΔpH can be varied in a different direction. Nigericin decreases pH_{in} and

hyperpolarizes $\Delta\psi_m$, while valinomycin elevates pH_{in} and depolarizes $\Delta\psi_m$. We measured $\Delta\psi_m$, pH_{in} and H_2O_2 production systematically, whereas ΔpH was calculated. To scrutinize Selivanov's theory, pH dependence of the above-mentioned parameters was also examined.

AIMS AND OBJECTIVES

During my dissertation I was interested in elucidating two main questions: (1) the effect of MB on the substrate level phosphorylation as a mechanism for ATP synthesis and (2) regulation of RET supported mtROS production due to *pmf.*

For the first objective of my thesis, we studied the effect of the compound MB, which has a mitochondrial target, on mitochondrial SLP catalysed by succinyl-CoA ligase on brain mitochondria isolated from guinea-pig cortex and we addressed the following questions:

- Does MB influence the mitochondrial SLP catalysed by succinyl-CoA ligase?
- How does MB influence the mitochondrial oxygen consumption, NAD(P)H autofluorescence and $\Delta\psi_m$ in ATP-synthase inhibited mitochondria respiring on different respiratory substrates?
- How does MB influence the mitochondrial oxygen consumption, NAD(P)H autofluorescence and $\Delta\psi_m$ in CI and ATP-synthase inhibited mitochondria? Is MB able to rescue $\Delta\psi_m$ after mitochondrial impairment?

For the second objective of the thesis, we investigated the effect of the components of *pmf*, namely $\Delta\psi_m$ and ΔpH, on a special case of mtROS production, called RET on brain and heart mitochondria isolated from guinea-pig. The following questions have been addressed:

- Which component of the *pmf* has a greater role in RET-supported H_2O_2 production in mitochondria fuelled by succinate and α-GP respiring mitochondria?
- How does pH_{extra} influence the H_2O_2 production supported by succinate and α-GP?
- How is ΔpH changed upon elevation of pH_{extra} in succinate and α-GP-respiring mitochondria?
- Is there a tissue specific regulation of RET-supported H_2O_2 production?

MATERIALS AND METHODS

Mitochondria were prepared from albino guinea pig brain cortex using a Percoll gradient and from whole heart using differential centrifugation as previously described. Animal experiments were performed in accordance with the Guidelines for Animal Experiments of Semmelweis University. A modified biuret method was used to determine protein concentration in the mitochondrial fraction.

Measurement of mitochondrial oxygen consumption

The mitochondrial oxygen consumption was determined with the polarographic High-Resolution Respirometry (O2k, Oroboros Instruments, Innsbruck, Austria).

Measurement of ATP production

To measure ATP production, a coupled enzyme assay with hexokinase and glucose-6-phosphate dehydrogenase was used. Briefly, ADP was phosphorylated to ATP in the presence of mitochondria and respiratory substrates. Then, hexokinase in the medium phosphorylated glucose to glucose-6 phosphate. Subsequently, glucose-6-phosphate was oxidised to 6-phosphogluconate by glucose-6-phospate dehydrogenase with the concomitant reduction of $NADP^+$ to NADPH. NADPH formation was detected as an indicator proportional to ATP release from the mitochondria.

Measurement of mitochondrial membrane potential

$\Delta\psi_m$ was assessed using safranin-O, a lipophilic cationic fluorescent dye, which accumulates in the mitochondrial membrane upon hyperpolarisation resulting in fluorescence quenching. Moreover, $\Delta\psi_m$ was also estimated *via* the distribution of tetraphenylphosphonium ion (TPP^+) with a custom-made TPP^+-selective electrode.

Measurement of hydrogen peroxide (H_2O_2) production

In the assay Amplex UltraRed® reacts with H_2O_2 in a 1:1 stoichiometry in the presence of horseradish peroxidase, producing the fluorophore UltroxRed. Fluorescence is then detected by a fluorimeter.

Measurement of intramitochondrial pH

Intramitochondrial pH (pH_{in}) of isolated mitochondria was measured with the acetoxymethyl ester form of 2,7-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF/AM). Upon entering in the mitochondrial matrix, the ester group is hydrolysed resulting in the formation of the pH-sensitive dye, BCECF.

Measurement of NAD(P)H autofluorescence and absorbance of methylene blue

NAD(P)H autofluorescence was detected with a fluorimeter at 344 nm excitation and 460 nm emission wavelengths. The oxidized and reduced form of MB was detected at 660 nm using a spectrophotometer.

RESULTS

I. Effect of methylene blue on mitochondrial substrate level phosphorylation in isolated brain mitochondria

In preliminary experiments, the addition of ADP to isolated mitochondria in an ATP assay test resulted in a consistent, long-lasting NAD(P)H+ H^+ production in the absence of respiratory substrates. This OXPHOS and SLP-independent ATP production may be attributed to the presence of AK. To eliminate its activity, an inhibitor of AK, 200 μM AP5, was used, which exerted a 83% inhibition of the AK-initiated ATP production. In order to detect the effect of MB on mitochondrial SLP, OXPHOS was also inhibited with the specific inhibitor of ATP-synthase, oligomycin.

In our study, the effect of 300 nM, 1 μM and 2 μM MB was investigated on mitochondrial oxygen consumption, ATP synthesis, $\Delta\psi_m$ and NAD(P)H autofluorescence in brain mitochondria respiring on different respiratory substrates (α-ketoglutarate [α-KG], succinate, malate and glutamate). First, ADP was added to mitochondria, then OXPHOS was initiated by the addition of the respiratory substrates. Next, oligomycin was added to inhibit ATP synthase, which was followed by the administration of MB. In α-KG or glutamate respiring mitochondria, MB increased the rate of ATP synthesis, whilst in succinate or malate energized mitochondria MB did not have any effect on the rate of ATP synthesis. This observation can be explained by the fact that succinate and malate enter the Krebs cycle downstream the reaction catalysed by succinyl-CoA ligase, therefore, these

substrates do not support the formation of succinyl-CoA, which is the substrate of succinyl-CoA ligase.

Oxygen consumption experiments followed a similar protocol as that of the ATP production measurements described in the previous paragraph. Respiratory substrates and ADP increased respiration, while oligomycin inhibited it. The subsequent addition of MB increased the respiration with each respiratory substrate. Under such conditions, due to the inhibition of the ATP-synthase by oligomycin, NADH accumulated, which in turns blocked the Krebs cycle. However, MB was capable of accepting electrons from NAD(P)H and $FADH_2$, providing an alternative pathway to transfer electrons to cytochrome c when the ETS was impaired. This alternative electron transfer mechanism bypasses CI, CII or CIII therefore changes the efficiency of proton pumping. In line with this, we could prove decreased NAD(P)H autofluorescence in α-KG supported, MB-treated mitochondria, as compared to controls.

The inner mitochondrial membrane is hyperpolarised in the presence of respiratory substrates, while ADP decreases $\Delta\psi_m$. Oligomycin increased mitochondrial membrane potential $\Delta\psi_m$ by inhibiting proton translocation via the ATP synthase, leading to accumulation of protons in the intermembrane space. However, MB addition did not modulate $\Delta\psi_m$ in the presence of oligomycin with the substrates α-KG, malate or glutamate. Collectively, $\Delta\psi_m$ could not be rescued by MB.

Neurodegenerative diseases like Parkinson's and Alzheimer's diseases are associated with mitochondrial dysfunction, in particular

with CI injury. The question arises whether MB can improve mitochondrial function by enhancing SLP in CI inhibition. To address this question, we have investigated the effect of MB on ATP synthesis in CI and ATP-synthase inhibited mitochondria. Under such circumstances, MB increased ATP synthesis in α-KG energized mitochondria, which could not be observed with malate. Both in α-KG and malate respiring mitochondria, ADP decreased $\Delta\psi_m$, oligomycin hyperpolarized the mitochondrial membrane, while rotenone (inhibitor of CI) depolarized it. The following addition of 2 μM MB rescued $\Delta\psi_m$ with both substrates.

II. Effect of the components of the proton motive force on mitochondrial membrane potential and transmembrane pH gradient on the reverse electron transfer supported H_2O_2 generation in brain and heart mitochondria

To study the effect of $\Delta\psi_m$ and ΔpH on the RET-evoked H_2O_2 production with succinate and α-GP in brain, the components of the *pmf* were dissected with the addition of ionophores such as nigericin and valinomycin. ADP was absent providing a high $\Delta\psi_m$ to support RET. To eliminate confounding factors that could have influenced ROS production (such as succinate transport or further metabolism of succinate by the Krebs cycle), not only succinate, but also α-GP was used to energize mitochondria and support RET-mediated ROS production. In order to gain a deeper insight into the effects of nigericin on RET, α-GP was also applied as a respiratory substrate. Unlike succinate, α-GP does not enter the mitochondria, it is oxidized

by α-GPDH on the outer surface of the inner mitochondrial membrane and does not form NADH.

Nigericin, as a K^+/H^+ antiporter, is responsible for the electroneutral exchange of K^+ and H^+. In our hands, nigericin hyperpolarized the mitochondrial membrane, increased H_2O_2 production, decreased pH_{in} and ΔpH both with succinate and α-GP in brain.

In the presence of valinomycin, the mitochondrial membrane is permeable to K^+, which led to a decrease of $\Delta\psi_m$ and H_2O_2 production and increase of pH_{in} and ΔpH.

In summary, our studies with the two ionophores showed that the RET-evoked H_2O_2 production always varied in accordance with changes of $\Delta\psi_m$ in brain. Conclusively, $\Delta\psi_m$ has a greater influence on mitochondrial RET-initiated H_2O_2 formation than does ΔpH.

We have also investigated the effect of pH_{extra} on RET-supported H_2O_2 production in succinate or α-GP respiring brain mitochondria. The pH of the respiration medium was adjusted before the experiments to 6.4, 6.8, 7.0, 7.2, 7.6, 8.0. We measured $\Delta\psi_m$, H_2O_2 production and pH_{in}, while ΔpH was calculated. Elevation of pH_{extra} resulted in increased mtROS formation, a finding that suggests a clear correlation between absolute pH and H_2O_2 production. However, there is inverse dependence between H_2O_2 generation and ΔpH: upon increase of pH_{extra}, H_2O_2 generation is higher, while ΔpH was lower. Based on the above observation, we can exclude ΔpH as a primary determinant of RET-evoked H_2O_2 production.

Detecting RET is also relevant in organs other than the brain. Heart mitochondria are often exposed to oxidative stress under pathological conditions, i.e. under ischemia-reperfusion. To deepen our understanding on RET in heart mitochondria, effects of ΔpH and $\Delta\psi_m$ on succinate-supported H_2O_2 production were investigated applying the above-mentioned ionophores. In our study, ionophores displayed a similar effect on ΔpH, $\Delta\psi_m$ and on H_2O_2 production both in brain and heart mitochondria. In conclusion, a tissue-specific regulation of the RET-supported H_2O_2 generation unlikely.

SUMMARY

I. Impairment of mitochondrial ETS is accompanied by insufficient ATP generation and elevated ROS production. Substrate level phosphorylation (SLP), which is catalysed by succinyl-CoA ligase in the citric acid cycle, is an alternative source of ATP production during mitochondrial dysfunction. In the first part of my thesis I elucidated the effects of the neuroprotective compound, methylene blue (MB) on SLP using brain mitochondria isolated from guinea pig. It is well-known that MB provides an alternative way of electron transport from NADH and $FADH_2$ to cytochrome c during complex I or complex III impairment.

Our new findings are the following:

- MB initiated mitochondrial SLP catalysed by succinyl-CoA ligase in α-KG and glutamate respiring mitochondria under conditions, when the other ATP producing enzymes, adenylate kinase and the ATP synthase were inhibited.
- MB increased the oligomycin-inhibited ATP synthesis in α-KG and glutamate energised mitochondria, but not in succinate and malate respiring mitochondria. Under the same conditions, MB did not influence $\Delta\psi_m$ in oligomycin-inhibited mitochondria regardless the substrates.
- MB increased the oxygen consumption with each substrate in oligomycin-inhibited mitochondria and decreased the NAD(P)H autofluorescence after the addition of oligomycin with α-KG.

- MB increased the oxygen consumption and restored $\Delta\psi_m$ in CI and ATP synthase inhibited, α-KG or malate respiring mitochondria.

II. The reverse electron transfer (RET) supported ROS is a special condition of mtROS formation, when electrons are transferred from CII and α-GP towards CI in hyperpolarised mitochondria. In the second part of the present work, we have investigated the effect of the components of the *pmf* ($\Delta\psi_m$ and ΔpH) on the mitochondrial H_2O_2 production, in particular, on RET-evoked H_2O_2 generation with succinate and α-GP. The aim was to determine which component of *pmf* displays the more dominant effect on RET-provoked H_2O_2 generation in isolated guinea pig brain and heart mitochondria respiring on succinate or α-GP.

Our findings are listed in the following points:

- $\Delta\psi_m$ has a greater role in the modulation of RET-evoked H_2O_2 production than ΔpH in succinate or α-GP respiring mitochondria.
- Upon elevation of pH_{extra}, RET-supported H_2O_2 formation was increasing in succinate or α-GP energised mitochondria.
- There is an inverse relationship between the RET-provoked H_2O_2 generation and ΔpH: upon elevation of $pH_{extra,}$ ΔpH decreased, while the H_2O_2 production increased.
- Our studies in heart mitochondria confirmed our findings in brain: $\Delta\psi_m$ has a greater role in the modulation of RET-evoked H_2O_2 production than ΔpH in succinate respiring mitochondria. Upon elevation of pH_{extra}, RET-supported H_2O_2 formation was

increasing in succinate energised mitochondria. Based on these observations tissue specific modulation of RET-evoked H_2O_2 generation via *pmf* in not likely.

ABBREVIATIONS

α-GP: alpha -glycerophosphate
α-GPDH: alpha -glycerophosphate dehydrogenase
AK: adenilate kinase
α-KG: alpha-ketoglutarate
α-KGDH: alpha-ketoglutarate dehidrogenase
ANT: adenin nukleotid translocase, ADP/ATP transporter
AP5: P^1,P^5-Di(adenosine-5′) pentaphosphate
BCECF-AM: 2',7'-bisz(2-karboxietil)-5,6karboxifluorescein acetoxi-methylester
CI: mitochondrial complex I,
CII: mitochondrial complex II, succinate dehidrogenase
CIII: mitochondrial complex III
CIV mitochondrial IV, citochrome c oxidase
ΔpH: transmembrane pH gradient
$\Delta\psi_m$: mitochondrial membrane potential
H_2O_2: hidrogen peroxide
MB: methylene blue
MBH_2: leucomethylene blue
mtROS: mitochondrial reactive oxygen species
OXPHOS: oxidative phosphorylation
pH_{extra}: extramitochondrial pH
pH_{in}: intramitochondrial pH
pmf: proton motive force
RET: reverse electron transfer
ROS: reactive oxygen species
SLP: substrate level phosphorlyation
TPP^+: triphenyl phosphonium ion

LIST OF PUBLICATIONS

Publications related to the present thesis:

Komlódi T, Tretter L. Methylene blue stimulates substrate-level phosphorylation catalysed by succinyl-CoA ligase in the citric acid cycle. Neuropharmacology. 2017 Sep 1;123:287-298. doi: 10.1016/j.neuropharm.2017.05.009.
Impact factor: 5.012 (2016/2017)

Komlódi T, Geibl FG, Sassani M, Ambrus A, Tretter L: Membrane potential and delta pH dependency of reverse electron transport-associated hydrogen peroxide production in brain and heart mitochondria
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Publications not related to the present thesis:

Koncsos G, Varga ZV, Baranyai T, Boengler K, Rohrbach S, Li L, Schlüter KD, Schreckenberg R, Radovits T, Oláh A, Mátyás C, Lux Á, Al-Khrasani M, Komlódi T, Bukosza N, Máthé D, Deres L, Barteková M, Rajtík T, Adameová A, Szigeti K, Hamar P, Helyes Z, Tretter L, Pacher P, Merkely B, Giricz Z, Schulz R, Ferdinandy P. Diastolic dysfunction in prediabetic male rats: Role of mitochondrial oxidative stress. Am J Physiol Heart Circ Physiol. 2016 Oct 1;311(4):H927-H943. doi: 10.1152/ajpheart.00049.2016
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Impact factor: 4.070 (2016)

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