Metabolic pathways affecting mitochondrial substrate-level phosphorylation

PhD thesis booklet

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Budapest
2018

INTRODUCTION

In normoxia, mitochondria can synthesize ATP for the cell via oxidative phosphorylation. However, in anoxia or in case of respiratory chain defects, this way of energy production is not functional. Under such pathological conditions mitochondria can turn from ATP-producing into ATP consuming organelles and through the reduction of cytosolic ATP pools they can aggravate the outcome of a given disease. Substrate-level phosphorylation catalyzed by succinate-CoA ligase has a critical role in preventing this, since the reaction enables the production of high energy phosphates independently from the respiratory chain. Thus as long as substrate-level phosphorylation is functional, mitochondria are able to export ATP from the matrix in spite of respiratory chain inhibition. In the reversible reaction catalyzed by succinate-CoA ligase succinyl-CoA, ADP (or GDP) and P_i is converted to succinate, ATP (GTP) and CoASH. Metabolic pathways influencing this reaction can be of special importance in determining to what extent this rescue mechanism can maintain the ATP-exporting mode of mitochondria in pathological conditions where oxidative phosphorylation is impaired.

One such pathway that might affect mitochondrial substrate-level phosphorylation is the metabolism of GABA via the GABA shunt. GABA is a neurotransmitter which is catabolized in the mitochondrial matrix to succinic semialdehyde (SSA), and finally to succinate which is able to enter the citric acid cycle. γ-Hydroxybutyrate (GHB) is a neurotransmitter and a psychoactive drug which is also converted to succinate during its degradation through the GABA shunt. In anoxia the further oxidation of the produced succinate by complex II is hindered, therefore matrix succinate concentration will be elevated. This can shift the equilibrium of the reversible reaction catalyzed by succinate-CoA ligase towards ATP hydrolysis. We hypothesized that operation of the GABA shunt inhibits mitochondrial substrate-level phosphorylation in anoxia this way, and thus mitochondria become ATP-consumers.

For the operation of succinate-CoA ligase an adequate succinyl-CoA provision by the α-ketoglutarate dehydrogenase complex (KGDHC) is needed. However, the reaction catalyzed by KHDHC requires oxidized NAD^+. When complex I is inhibited, NADH oxidation

can be performed by mitochondrial diaphorases, reducing suitable quinone substrates at the same time. From the reduced quinone compound the electrons can be transferred to the respiratory chain. Previous experiments in our laboratory have shown that the endogenous quinone pool available in the matrix is sufficient for the reaction to proceed. However, diaphorase enzymes are not saturated with these quinones, and when the $NADH/NAD^+$ ratio is artificially elevated, the addition of exogenous quinone substrates can greatly boost substrate-level phosphorylation. The second major issue of the present research aimed at examining the contribution of a diaphorase enzyme and potential diaphorase substrates to NADH oxidation and thus to substrate-level phosphorylation.

OBJECTIVES

The first question raised in the present work is whether the operation of the GABA shunt in anoxia shifts the reaction mediated by succinate-CoA ligase into ATP consuming direction due to the accumulation of succinate. To test this hypothesis we examined the effects of exogenous GABA, SSA and GHB addition on the bioenergetic parameters and substrate-level phosphorylation of isolated mitochondria.

The identity of the diaphorases participating in NAD^+ regeneration for substrate-level phosphorylation in case of complex I inhibition is not known. The second aim of this thesis is to address the contribution of a diaphorase enzyme, NAD(P)H quinone oxidoreductase 1 (Nqo1) to NADH oxidation under conditions of impaired mitochondrial respiration.

Finally, we intended to test five quinone compounds (menadione, mitoquinone, duroquinone, idebenone and 2-methoxy-1,4-naphtoquinone) as possible diaphorase substrates. We wished to examine if these quinones indeed support substrate-level phosphorylation by providing NAD^+ and whether they exert this potential effect through Nqo1.

MATERIALS AND METHODS

Animals

Wild type and Nqo1$^{-/-}$ mice were of mixed 129Sv and C57Bl/6 background and between 2 and 6 months of age.

Isolation of mitochondria

Mouse brain and liver mitochondria were isolated via differential centrifugation. In case of brain non-synaptic mitochondria were purified on a Percoll gradient. Protein concentration of the samples was determined using the BCA (bicinchoninic acid) assay using a Tecan Infinite® 200 PRO series plate reader.

Determination of membrane potential in isolated mitochondria

Membrane potential of isolated mitochondria was estimated in a Hitachi F-7000 spectrofluorimeter or Oroboros Oxygraph-2k using a fluorescent dye, safranine O. The fluorescence signal was converted to millivolts using a voltage-fluorescence calibration curve. Experiments were performed at 37 °C.

Mitochondrial respiration

Oxygen consumption of mitochondria was measured polarographically, parallel with the membrane potential, in an Oroboros Oxygraph-2k instrument. Oxygen concentration and flux were recorded using the DatLab software. Experiments were performed at 37 °C.

Determination of NADH autofluorescence in isolated mitochondria

Mitochondrial NADH autofluorescence was measured at 37 °C using a Hitachi F-7000 fluorescence spectrophotometer. NADH autofluorescence was calibrated by adding known amounts of NADH to the suspension.

Determination of diaphorase activity

Diaphorase activity was measured by two different methods in the cytosolic and mitochondrial fractions of wild type and Nqo1$^{-/-}$ mice. Cytosolic fractions were obtained by ultracentrifugation of the liver homogenate. The first method relies on the reduction of a redox dye, 2,6-dichlorophenol-indophenol (DCPIP), with NADH or NADPH as electron donor. Reduction of DCPIP is

followed spectrophotometrically. In the second method electrons are transferred by diaphorases from NADH to a quinone, and finally a secondary electron acceptor, cytochrome c is reduced, which can be detected spectrophotometrically. Activities were determined by either method in the presence of a diaphorase inhibitor, dicoumarol as well. All assays were performed at 30 °C.

Cell culturing

HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum and antibiotic solution at 37 °C in 5% CO_2.

Mitochondrial membrane potential determination of in situ mitochondria of permeabilized HepG2 cells

HepG2 cells were permeabilized by digitonin, and mitochondrial membrane potential ($\Delta\Psi_m$) was estimated in a Tecan Infinite® 200 PRO series plate reader using the fluorescent dye safranine O. Experiments were performed at 37 °C.

Transfections of HepG2 cells

HepG2 cells were transfected with siRNA specific to human *NQO1* or scrambled siRNA, using lipofectamine 2000. Cells were probed for mitochondrial SLP after 56 hours, and immediately afterwards harvested for Western blotting

Western blotting

HepG2 cells were solubilized in RIPA buffer containing a cocktail of protease inhibitors and were frozen at −80°C for further analysis. Frozen pellets were thawed on ice, and their protein concentration was determined using the BCA (bicinchoninic acid) assay. Samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Separated proteins were transferred onto a methanol-activated polyvinylidene difluoride membrane. Immunoblotting was performed using rabbit polyclonal anti-NQO1 and mouse monoclonal anti-β-actin primary antibodies. Immunoreactivity was detected using the appropriate peroxidase-linked secondary donkey antibody and enhanced chemiluminescence detection reagent.

RESULTS

Catabolism of GABA, succinic semialdehyde or γ-hydroxybutyrate through the GABA shunt impairs mitochondrial substrate-level phosphorylation

To test the hypothesis that the conversion of GABA, SSA and GHB to succinate inhibits substrate-level phosphorylation, first we wished to verify that the three compounds can be metabolized in isolated mouse brain and liver mitochondria. Addition of GABA, SSA and GHB resulted in membrane potential generation in both brain and liver mitochondria, except for when GHB was added to brain mitochondria. From the three compounds SSA was the most efficient substrate energizing mitochondria, it was able to build up the maximum membrane potential. This polarization was not ceased by separate inhibition of complex I or complex II, but membrane potential collapsed when the inhibitors of both complexes were present. This implies that proton-motive force generated by SSA is supported by both $FADH_2$ and NADH production. When we recorded the effect of the substrate on NADH autofluorescence, the extent of NADH production by SSA was approximately 5 times higher in liver than in brain mitochondria.

Substrate-level phosphorylation can be investigated by interrogating the directionality of the adenine nucleotide translocase (ANT). When the respiratory chain is inhibited, but substrate-level phosphorylation is still operational, the ANT will continue exporting ATP from mitochondria. However, when substrate-level phosphorylation is inhibited, the ANT will reverse and import extramitochondrial ATP into the matrix. The directionality of the transporter can be deduced from the change in membrane potential evoked by an ANT inhibitor, carboxyatractyloside. In the presence of substrate combinations that support substrate-level phosphorylation (glutamate and malate or alpha-ketoglutarate and malate), the ANT did not reverse after reaching anoxia but was still transporting ATP out of the matrix. When the experimental medium contained GABA, SSA or GHB as well, this resulted in reversal of the ANT in anoxia, implying the inhibition of substrate-level phosphorylation. Again, exceptions from this were the experiments performed on brain mitochondria in the presence of GHB, since here GHB did not have any effect on substrate-level phosphorylation.

Contribution of Nqo1 to mitochondrial substrate-level phosphorylation using endogenous or exogenous quinones

To examine the role of Nqo1 in NAD^+ provision we compared samples from the livers of wild type and $Nqo1^{-/-}$ mice. NADH-oxidizing activity using DCPIP as electron acceptor was much smaller in the cytosolic fraction of the knockout sample than in the wild type. On the contrary, when diaphorase activity of the mitochondrial fraction was measured, no statistically significant difference was found between the two types of samples. Using menadione, duroquinone or 2-methoxy-1,4-naphtoquinone as primary electron acceptor, the knockout mitochondrial fraction exhibited lower diaphorase activity compared to the wild type, which difference reached statistical significance in the case of 2-methoxy-1,4-naphtoquinone. Mitochondrial respiration and substrate-level phosphorylation in anoxia or during complex I inhibition were not altered in the knockout mitochondria. From these experiments we concluded that Nqo1 is dispensable for the operation of substrate-level phosphorylation during respiratory

inhibition. Nevertheless, diaphorase inhibitors impeded substrate-level phosphorylation in the Nqo1$^{-/-}$ samples.

We tested whether the five quinone compounds - after being reduced by diaphorases using NADH - can donate electrons to the respiratory chain. The examined quinones increased mitochondrial respiration, and lead to membrane potential generation in the wild type samples when complex I was inhibited by rotenone. To investigate if the quinones support substrate-level phosphorylation via NADH oxidation, mitochondria were energized by a substrate combination that elevates the intramitochondrial NADH/NAD$^+$ ratio. From the five quinones three: duroquinone, idebenone and 2-methoxy-1,4-naphtoquinone enhanced mitochondrial substrate-level phosphorylation in the presence of rotenone. From these 2-methoxy-1,4-naphtoquinone exerted its effect only in the wild type sample, in the Nqo1$^{-/-}$ mitochondria it did not influence substrate-level phosphorylation. The effect of 2-methoxy-1,4-naphtoquinone was abolished by diaphorase inhibitors. In anoxia only duroquinone enabled substrate-level phosphorylation to proceed, and this was observed in the wild type as well as in the knockout samples. We examined the effect of

duroquinone, idebenone and 2-methoxy-1,4-naphtoquinone in permeabilized HepG2 cells – a cell line which is known to express NQO1 at high levels. These three quinones supported substrate-level phosphorylation in a dicoumarol-sensitive manner. To confirm that 2-methoxy-1,4-naphtoquinone contributes to the maintenance of intramitochondrial ATP production as a substrate of NQO1, we transfected HepG2 cells with siRNA directed against the enzyme. However, as shown by western blot analysis NQO1 protein expression was diminished only to a small extent. In line with this, 2-methoxy-1,4-naphtoquinone had the same effect in the in situ mitochondria of siRNA-transfected HepG2 cells as in case of the control cells.

CONCLUSION

Our results show that GABA, SSA and GHB are metabolized in isolated mouse brain and liver mitochondria, except for GHB in the brain samples. SSA proved to be an extremely efficient substrate energizing mitochondria, through the production of NADH and FADH2. In anoxia, the conversion of the molecules through the GABA shunt impairs mitochondrial substrate-level phosphorylation.

From the experiments performed on $Nqo1^{-/-}$ samples we concluded that the contribution of Nqo1 to mitochondrial NADH oxidation is small, and the enzyme is not necessary for the operation of substrate-level phosphorylation during respiratory chain inhibition. However, our results imply that other, still unknown dicoumarol-sensitive diaphorases are indispensable for the reaction. From the examined quinones in anoxia only duroquinone, during complex I inhibition duroquinone, idebenone and 2-methoxy-1,4-naphtoquinone as well are able to support substrate-level phosphorylation. 2-Methoxy-1,4-naphtoquinone exerts this beneficial effect as a substrate for Nqo1.

LIST OF PUBLICATIONS

Publications related to the PhD thesis

Nemeth B, Doczi J, Csete D, Kacso G, Ravasz D, Adams D, Kiss G, Nagy AM, Horvath G, Tretter L, Mocsai A, Csepanyi-Komi R, Iordanov I, Adam-Vizi V, Chinopoulos C. (2016) Abolition of mitochondrial substrate-level phosphorylation by itaconic acid produced by LPS-induced Irg1 expression in cells of murine macrophage lineage. Faseb j, 30: 286-300

IF.: 5.498

Kacso G, Ravasz D, Doczi J, Nemeth B, Madgar O,Saada A, Ilin P, Miller C, Ostergaard E, Iordanov I, Adams D, Vargedo Z, Araki M, Araki K, Nakahara M, Ito H, Gal A, Molnar MJ, Nagy Z, Patocs A, Adam-Vizi V, Chinopoulos C. (2016) Two transgenic mouse models for beta-subunit components of succinate-CoA ligase yielding pleiotropic metabolic alterations. Biochem J, 473: 3463-3485.

IF.: 3.797

Ravasz D, Kacso G, Fodor V, Horvath K, Adam-Vizi V, Chinopoulos C. (2017) Catabolism of GABA, succinic semialdehyde or gamma-hydroxybutyrate through the GABA shunt impair mitochondrial substrate-level phosphorylation. Neurochem Int, 109:41-53.

IF.: 3.603

Ravasz D, Kacso G, Fodor V, Horvath K, Adam-Vizi V, Chinopoulos C. (2018) Reduction of 2-methoxy-1,4-naphtoquinone by mitochondrially-localized Nqo1 yielding NAD+ supports substrate-level phosphorylation during respiratory inhibition. Biochim Biophys Acta, 1859: 909-924.

IF.: 4.280

Publications not related to the PhD thesis

Chen E, Kiebish MA, McDaniel J, Gao F, Narain NR, Sarangarajan R, Kacso G, Ravasz D, Seyfried TN, Adam-Vizi V, Chinopoulos C. (2016) The total and mitochondrial lipidome of Artemia franciscana encysted embryos. Biochim Biophys Acta, 1861:1727-1735.

IF.: 5.547

Chen E, Kiebish MA, McDaniel J, Niedzwiecka K, Kucharczyk R, Ravasz D, Gao F, Narain NR, Sarangarajan R, Seyfried TN, Adam-Vizi V, Chinopoulos C. (2018) Perturbation of the yeast mitochondrial lipidome and associated membrane proteins following heterologous expression of Artemia-ANT. Sci Rep, 8:5915.

IF.: 4.122