The contribution of the reaction catalyzed by succinyl-CoA ligase to substrate-level phosphorylation

PhD Thesis Booklet

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Budapest
2018

INTRODUCTION

The “powerhouse” of cells are mitochondria. In the last few decades, it has been highlighted that impaired mitochondrial function is associated with several pathological conditions, such as excitotoxicity, ischemia/reperfusion-, neurodegenerative diseases, and oxidative stress.

Energy limiting conditions lead to dysfunction of these organelles through losing inner mitochondrial membrane impermeability and/or losing mitochondrial membrane potential ($\Delta\Psi m$). A serious dysfunction accompanied by complete depolarization can lead to cytosolic ATP pool depletion. It is not surprising that has to be a rescue mechanism that is able to support to maintain the cellular ATP pool in those cells, where ATP synthesis through the oxidative phosphorylation is not satisfactory. A possible rescue mechanism revealed by our laboratory is the mitochondrial substrate-level phosphorylation catalyzed by succinyl-CoA ligase and other succinyl-CoA ligase supporting enzymes.

Mitochondrial diseases are collectively considered to be a primary cause of encephalomyopathies and other multisystem maladies. A sizeable fraction of this pool of diseases are associated with mtDNA depletion. Many animal

models have been generated to model mtDNA depletion by explicitly deleting genes essential for mtDNA replication, although only one study has addressed the role of succinyl-CoA ligase.

OBJECTIVES

1. Set up animal models for succinyl-CoA ligase deficiencies and investigate the impact of the mitochondrial substrate-level phosphorylation on the bioenergetics parameters.

2. Advise the following questions:
    a. How and what extent do succinyl-CoA ligase mutations affect substrate-level phosphorylation?
    b. What happens if the SKDCC axis (alpha-ketoglutarate dehydrogenase complex, DT-diaphorases, complex III and cytochrome c), the primary, central enzyme, the succinyl-CoA ligase itself was damaged?
    c. What would be the difference between ATP- or GTP-forming subunit affecting mutations?

3. Using this animal model, reveal and elucidate the details of known symptoms, alteration in metabolites and mtDNA content in order to contribute the better understanding of the succinyl-CoA ligase mutation and the function of the succinyl-CoA ligase enzyme.

4. Reveal a potential compensatory mechanism which is able to prevent phenotypic alterations in the heterozygous patients and animal model.

METHODS

Animals

Mice were of either 129/SvEv (*Sucla2* heterozygote strain) or C57Bl/6N (*Suclg2* heterozygote strain) background.

Sucla2+/− heterozygous mice were generated by Texas A&M Institute for Genomic Medicine (TIGM) using a gene-trapping.

Suclg2 heterozygote mice [B6-Suclg2Gt(pU-21KBW)131Card] were generated at CARD, Kumamoto University, Japan also using a gene-trapping technique Neither *Sucla2* −/− nor *Suclg2* −/− mice were ever born from mating heterozygous mice, suggesting that complete

absence of either gene is incompatible with life in mice. By mating *Sucla2* heterozygous mice with *Suclg2* heterozygous mice, double transgenic (*Sucla2*+/−/*Suclg2*+/−) mice were born and viable.

Isolation of mitochondria

Isolation of mitochondria from mouse liver, heart, and brain: liver and heart mitochondria from all animals were isolated with a differrential centrifugation method. Nonsynaptic brain mitochondria were isolated on a Percoll gradient. Protein concentration was determined using the bicinchoninic acid assay.

Determination of protein concentration

Protein concentration was determined using the bicinchoninic acid assay (ThermoFisher SCIENTIFIC - Pierce™ BCA Protein Assay Kit), and calibrated using bovine serum albumin standards using a Tecan Infinite® 200 PRO series plate reader (Tecan Deutschland GmbH, Crailsheim, Germany).

Determination of membrane potential (ΔΨm) in isolated liver mitochondria

Mitochondrial membrane potential (ΔΨm) of isolated mitochondria was estimated using fluorescence quenching of safranin O. The cationic dye can accumulate inside the negatively charged matrix of energized mitochondria. Fluorescence was recorded in either a Hitachi F-4500 fluorescence spectrophotometer or using the O2k-Fluorescence LED2-Module of the OROBOROS Oxygraph-2k. To convert safranin O fluorescence into millivolts, a voltage-fluorescence calibration curve was constructed.

Mitochondrial respiration

Oxygen consumption was estimated polarographically using an Oxygraph-2k. 0.5-1 mg -depending on the tissue of origin- mitochondria was suspended in 2 ml incubation medium, the composition of which was identical to that for ΔΨm determination. Experiments were performed at 37 ^{o}C. Oxygen concentration and oxygen flux (pmol·s−1·mg−1; negative time derivative of oxygen concentration, divided by mitochondrial mass per volume and corrected for instrumental background oxygen flux arising from oxygen consumption of the oxygen sensor

and back-diffusion into the chamber) were recorded using DatLab software (Oroboros Instruments).

Cell cultures

Fibroblast cultures from skin biopsies from the patient with no SUCLA2 expression and a control subject were prepared. Cells were grown on poly-L-ornithine coated flasks for 5-7 days in RPMI1640 medium supplemented with 10% fetal bovine serum and 2 mM glutamine and kept at 37 ^{o}C in 5% CO_2. The medium was also supplemented with penicillin, streptomycin, and amphotericin.

Mitochondrial membrane potential (ΔΨm) determination in *in situ* mitochondria of permeabilized fibroblast cells

ΔΨm was estimated using fluorescence quenching of the cationic dye safranine O due to its accumulation inside energized mitochondria. Fibroblasts were harvested by trypsinization, permeabilized and suspended in a medium identical to that as for ΔΨm measurements in isolated mitochondria. Substrates were 5 mM glutamate and 5 mM malate. Fluorescence was recorded in a Tecan Infinite®

200 PRO series plate reader. Experiments were performed at 37 ^{o}C.

Western blot analysis

Isolated mitochondria were solubilized in RIPA buffer containing a cocktail of protease inhibitors and frozen. Frozen pellets were thawed on ice, their protein concentration was determined, loaded at a concentration of 3.75 µg per well on the gels and separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred to a methanol-activated polyvinylidene difluoride membrane. Immunoblotting was performed as recommended by the manufacturers of the antibodies. Rabbit polyclonal anti-SUCLG1, anti-SUCLG2, anti-VDAC1 and anti-SUCLA2 primary antibodies were used. Immunoreactivity was detected using the appropriate peroxidase-linked secondary antibody (donkey anti-rabbit) and enhanced chemiluminescence detection reagent. Densitometric analysis of the bands was performed in Fiji software.

mtDNA content

Total DNA was isolated from 4 pooled tissues from each mouse group using QIAamp DNA Mini Kit (QIAGEN)

following the manufacturer's instructions. Relative mtDNA content was quantified in triplicate by real-time PCR using primers for cox1 and normalized against the nuclear encoded actinB gene. DNA was amplified in an ABI 7900 system.

Electron transport chain complex and citrate synthase activity assays

Enzymatic activities of rotenone-sensitive NADH CoQ reductase (complex I), succinate cytochrome c reductase (complex II/III), succinate dehydrogenase (complex II, SDH), cytochrome c oxidase (COX, complex IV) and citrate synthase (CS), a mitochondrial marker enzyme, were determined in isolated mitochondria.

Determination of succinyl-CoA ligase activity

ATP- and GTP-forming succinyl-CoA ligase activity in isolated mitochondria was determined at 30 °C using DTNB. Rates of 2-nitro-5-thiobenzoate formation were followed spectrophotometrically during constant stirring.

Determination of acylcarnitines

Multiple reaction monitoring transitions of butyl ester derivatives of acylcarnitines from dry blood spots and

stable isotope internal standards were analyzed by electrospray ionization-tandem mass spectrometry (MS-MS).

Determination of *Sucla2* mRNA by qRT-PCR

mRNA coding for *Sucla2* was quantified by qPCR in two different laboratories using two different 'housekeeping' mRNAs for normalization, ß-actin or proteasome 26S subunit, ATPase 4 (Psmc4). In both cases, total RNA was isolated from the organs (livers, hearts, brains) of at least four mice per age group and genotype (wild type or *Sucla2*+/−) with RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

RESULTS

Sucla2 mRNA quantification, succinyl-CoA ligase subunit expression and enzymatic activities in wild type vs Sucla2+/− mice

Mutant Sucla2 mice were generated using a gene-trapping technique.

Total RNA was isolated from the livers, hearts and brains of 3-, 6-, and 12 months old wild type and Sucla2+/− mice (four animals per group), and Sucla2 mRNA was quantified by qPCR, ratioed to β-actin or Psmc4 expression. mRNA coding for Sucla2 was significantly decreased (26-71%) in the tissues obtained from Sucla2+/− mice, compared to those obtained from wild type littermates. These results are in accordance to those obtained from immunodetection of Sucla2 subunit by Western blotting. As per our Western Blot results, Sucla2+/− mice exhibited up to 76% decrease in Sucla2 expression, depending on the tissue and the age of the mice. Concomitantly, Sucla2+/− mice exhibited up to 66% reduction in Suclg1 protein, but also up to 177% increase in Suclg2 protein.

In agreement with the above results regarding Suclg1/g2/a2 subunit quantification, ATP-forming activity of Sucla2+/− mice decreased, while GTP-forming activity increased, though only in heart mitochondria, for all ages.

Characterization of succinyl-CoA ligase subunit expression and enzymatic activities of wild type vs Suclg2+/− mice

Suclg2+/− mice exhibited an up to 56% decrease in Suclg2 expression, a mostly insignificant decrease in Suclg1 expression, and no rebound increase in Sucla2 expression. The variability in the decrease in Suclg2 (and Sucla2) expression in these transgenic mouse lines probably reflects the 'leakiness' of the mutant allele that could produce wild-type mRNAs by alternative splicing around the gene trap cassette.

Characterization of succinyl-CoA ligase subunit expression and enzymatic activities of Sucla2+/−/Suclg2+/− double heterozygote mice

We cross-bred Sucla2+/− mice with Suclg2+/− mice, which yielded viable Sucla2+/−/Suclg2+/− offsprings.

The results of Western blotting of mitochondria isolated from the brains, livers, hearts of 12 months old wild type vs Sucla2+/−/Suclg2+/− mice probing for SUCLG1, SUCLG2 and SUCLA2 (and VDAC1 as loading control) are the following: The deletion of one Sucla2 allele still yields a rebound increase in Sucgl2 expression in liver, albeit protracted because these mice also lack one Suclg2 allele. By the same token, the anticipated decrease (due to deletion of one Suclg2 allele) in Suclg2 expression is lost, presumably because of the effect(s) of deletion of the Sucla2 allele, antagonizing the diminution in expression of Suclg2. These results are also reflected from the measured ATP- and GTP-forming activities of wild type vs Sucla2+/−/Suclg2+/− mice: ATP-forming activity is diminished in the double heterozygote mice compared to wild type littermates due to loss of one Sucla2 allele, however, GTP-forming activity remains unaffected, despite the loss of one Suclg2 allele.

The effect of deleting one Sucla2 allele on mitochondrial respiration

Except for one combination for state 2 respiration and five combinations for state 3 respiration, the remaining 84

combinations of substrates per tissue of origin per age of mice did not reveal statistically significant differences between wild type and Sucla2+/− mice.

The effect of deleting one Sucla2 allele on ΔΨm and substrate-level phosphorylation during inhibition of complex I by rotenone or true anoxia

We evaluated matrix substrate-level phosphorylation during either inhibition of complex I by rotenone or during anoxia. There were no differences between mitochondria from wild type and Sucla2+/− mice. Likewise, when substrate-level phosphorylation was examined during inhibition of the respiratory chain by rotenone instead of anoxia, no differences between wild type and Sucla2+/− mice mitochondria were observed. By the same token, no differences in mitochondrial respiration or substrate-level phosphorylation during chemical or true anoxia was observed by comparing Suclg2+/− versus wild type littermate mice. By comparing wild type vs Sucla2+/−/Suclg2+/− double heterozygote mice, we also observed no difference in the ability of substrate-level phosphorylation to maintain ANT in the forward mode, except for using glutamate + malate + β-hydroxybutyrate

as substrates (a substrate combination that does not favor substrate-level phosphorylation) in liver mitochondria, where we obtained the full spectrum of results, ranging from maintenance of substrate-level phosphorylation, to its abolition. However, in permeabilized fibroblasts from a control subject vs a patient suffering from complete deletion of SUCLA2, the *in situ* mitochondria from the patient are unable to perform substrate-level phosphorylation during respiratory inhibition by rotenone.

The effect of deleting one Sucla2 allele on electron transport chain enzymes/citrate synthase

We investigated the effect of deleting one Sucla2 allele in mice on complex I, II, II/III and IV activities, ratioed to citrate synthase activity. As per our results, mitochondria from all tissues and all ages revealed no statistically significant differences between wild type and Sucla2+/ mice. However, by comparing wild type vs Sucla2+/−/Suclg2+/− double heterozygote mice, there was a statistically significant increase in succinate dehydrogenase activity in heart mitochondria.

The effect of deleting one Sucla2 allele on mtDNA

Because of the involvement of succinyl-CoA ligase in the maintenance of mtDNA, we compared the amount of mtDNA in the tissues of wild type vs Sucla2+/− mice. Relative mtDNA content from the livers, hearts and brains of 3-, 6- and 12 months old mice was quantitated by real time-PCR. There was a moderate but statistically significant decrease in mtDNA in all tissues of 3 months old mice, and in the brains of 12 months old mice. Furthermore, by comparing wild type vs Sucla2+/−/Suclg2+/− double heterozygote mice, there was a much greater statistically significant decrease in mtDNA in the livers and brains of double heterozygote mice, compared to wild-type littermates.

The effect of deleting one Sucla2 allele on blood carnitine esters

We measured the levels of 20 carnitine esters in the blood of mice. As per our results, there were statistically significant increases in 36 out of 63 comparisons of carnitine esters in the blood of Sucla2+/− mice from all age groups compared to that from wild type mice, but also

6 occasions in which carnitine esters of Sucla2+/− mice is decreased compared to those of wild type mice.

CONCLUSION

The most important finding of this thesis is, that the upregulation of Suclg2 in Sucla2+/– mice can compensate the severity of the phenotype of this genetic modification. However, Suclg2 one-allele deletion was not accompanied by Sucla2 upregulation. From the experiments with Sucla2+/– mice we obtained the information that deletion of one Sucla2 allele is associated with a decrease in Suclg1 expression and a rebound increase in Suclg2 expression. Furthermore, this is reflected in reciprocal decrease vs. increase in ATP-forming vs. GTP-forming succinyl-CoA ligase activity. However, from the experiments with Suclg2+/– mice, we concluded that deletion of one Suclg2 allele was not associated with the rebound effects as seen in the Sucla2+/− mice. A plausible explanation of this finding is that the Suclg2 is responsible for compensatory mechanisms and the upregulation of the one residual Suclg2 allele is so strong that it can compensate the loss of the knocked-out allele. We also could see in the experiments with Sucla2+/–/Suclg2+/– mice that the anticipated decrease (due to deletion of one Suclg2 allele) in Suclg2 expression is lost, presumably because of the effect(s) of deletion of the

Sucla2 allele, antagonizing the diminution in the expression of Suclg2. We concluded that the effect of deleting one Sucla2 allele up-regulating Suclg2 expression is so dominant that it adequately antagonizes or even supersedes the effect(s) of a concomitant loss of one Suclg2 allele.

Regarding the fact that succinyl-CoA ligase is at the intersection of several metabolic pathways it is surprising that SUCLA2 heterozygous patients are asymptomatic. According to our data regarding bloodspot analyses, we found a phenotypic alteration in heterozygous mice, namely in acyl-carnitine levels. These results are suggesting, that heterozygous patient with only one SUCLA2 mutant allele may also show changes in blood acyl-carnitines.

With our study we could profile the metabolism of two transgenic mouse models for β-subunit components of succinyl-CoA ligase and highlight a compensatory mechanism. The fact that in those patients where SUCLA2 or SUCLG2 mutations were found showed milder severity compared to those where the invariant α subunit was affected, also supports the notion that α subunit is responsible for maintaining both ATP- and GTP-forming activities. We further concluded from these results that the loss of the alpha subunit cannot be compensated by the upregulation of any other subunit.

BIBLIOGRAPHY

Publications related to the present thesis

Abolition of mitochondrial substrate-level phosphorylation by itaconic acid produced by LPS-induced Irg1 expression in cells of murine macrophage lineage.
Németh B, Doczi J, Csete D, **Kacso G**, Ravasz D, Adams D, Kiss G, Nagy AM, Horvath G, Tretter L, Mócsai A, Csépányi-Kömi R, Iordanov I, Adam-Vizi V, Chinopoulos C. FASEB J. 2016 Jan;30(1):286-300. doi: 10.1096/fj.15-279398.
Impact factor: 5.498

Two transgenic mouse models for β-subunit components of succinate-CoA ligase yielding pleiotropic metabolic alterations.
Kacso G, Ravasz D, Doczi J, Németh 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, Gál A, Molnár MJ, Nagy Z, Patocs A, Adam-Vizi V, Chinopoulos C.
Biochem J. 2016 Oct 15;473(20):3463-3485.
Impact factor: 3.797

Catabolism of GABA, succinic semialdehyde or gamma-hydroxybutyrate through the GABA shunt impair mitochondrial substrate-level phosphorylation.
Ravasz D, **Kacso G**, Fodor V, Horvath K, Adam-Vizi V, Chinopoulos C.
Neurochem Int. 2017 Mar 11. pii: S0197-0186(17)30045-1.
Impact factor: 3.262

Publications not related to the present thesis

The total and mitochondrial lipidome of Artemia franciscana encysted embryos.
Chen E, Kiebish MA, McDaniel J, Gao F, Narain NR, Sarangarajan R, **Kacso G**, Ravasz D, Seyfried TN, Adam-Vizi V, Chinopoulos C.
Biochim Biophys Acta. 2016 Nov;1861(11):1727-1735.
Impact factor: 5.547

Reduction of 2-methoxy-1,4-naphtoquinone by mitochondrially-localized Nqo1 yielding NAD+ supports substrate-level phosphorylation during respiratory inhibition
Ravasz D; **Kacso G**; Fodor V; Horvath K; Adam-Vizi V; Chinopoulos C
Biochim Biophys Acta. 2018 Sep;1859(9):909-924.
doi: 10.1016/j.bbabio.2018.05.002. Epub 2018 May 7.
Impact factor: 4.280