Bioenergetic consequences of heterozygous alpha-ketoglutarate dehydrogenase complex mutation

Ph.D. thesis

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1. Introduction

Mitochondria are an obligatory part of the eukaryotic multicellular life. These organelles play a crucial role in ATP production and in redox homeostasis. Moreover, the generation and elimination of reactive oxygen species (ROS) are important functions in cellular redox signalling. α-ketoglutarate dehydrogenase complex (KGDHc) catalyses the oxidative decarboxylation of α-ketoglutarate (α-KG) to succinyl-CoA, while nicotinamide adenine dinucleotide (NAD^+) is reduced to $NADH+H^+$ and a CO_2 is released. The enzyme complex is composed of three types of subunits, namely α-ketoglutarate dehydrogenase (KGDH, E1k), dihydrolipoyl succinyltransferase (DLST, E2k), and dihydrolipoyl dehydrogenase (DLD, E3). Homozygous knock-out of each subunit is lethal *in utero* pointing out the vital function of the KGDHc.

The E3 subunit generates a high amount of ROS under any condition in which the $NADH/NAD^+$ ratio is increased such as ischemic reperfusion injury. Importantly, KGDHc is not only a source, but also a target of ROS. A prolonged prooxidant environment can damage the complex, leading to its (ir)reversible inactivation. Moreover, loss of KGDHc activity has been observed in neurodegenerative disorders, such as Alzheimer's disease, Parkinson's disease, or Huntington's disease. Additionally, studies using single heterozygous $DLST^{+/-}$ and $DLD^{+/-}$ animals showed no significant phenotypic alterations compared to wild type littermates unless the rodents were exposed to an additional toxin (e.g., the Parkinson inducer neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPTP). Here, we want to prove that double heterozygous knock-out of $DLST^{+/-}$ and $DLD^{+/-}$ can lead to behavioural abnormalities and exacerbate mild illnesses.

2. Objectives

Our aim was to investigate the metabolic consequences of KGDHc dysfunction *in vivo* and *in vitro* using DLST and DLD double heterozygous knockout ($DLST^{+/-}/DLD^{+/-}$, DKO) mice strains. Our specific questions were as follows:

- How does *DKO* of the KGDHc influence mitochondrial bioenergetics such as mitochondrial oxygen consumption, ATP production and ROS production in isolated mitochondria?
- Do DKO mice possess altered endurance in a fatigue test?
- Is this genetical modification accompanied by any cognitive deficit and neurological alterations?
- Are there any histological alterations of different tissues between DKO and wild-type (WT) mice?
- Are there any age-dependent differences in mitochondrial bioenergetics, metabolic parameters and neurological alterations in these DKO mice strains compared to WT animals?

3. Methods

3.1. Animals

Double heterozygous $DLD^{+/-}/DLST^{+/-}$ mice were generated in-house by crossbreeding $DLD^{+/-}$ and $DLST^{+/-}$ strains. Male mice aged 100–150 days (WT100, DKO100) and 200–250 days (WT200, DKO200) were used in all experiments. All procedures were approved by the Animal Care and Use Committee of the Institute of Experimental Medicine and the Animal Health and Food Control Station, Budapest (permission numbers: PE/EA/00957-6/2024 and PE/EA/00960-4/2024).

3.2. *In vitro* measurements

Mitochondria were isolated from mice brain and kidney. The tissues were minced and homogenized on ice, then centrifugated multiple times to remove most non-mitochondrial fractions of cells. For the preparation of brain mitochondria, a discontinuous Percoll-gradient was also used. Mitochondria from both organs were resuspended in ice-cold medium and protein concentration was determined using a modified Biuret assay.

Oxygen consumption was measured using a polarographic oxygen sensor in a closed chamber with continuous stirring at 37 °C. The ATP production rate was determined using a coupled-enzyme assay based on hexokinase and glucose-6-phosphate dehydrogenase, with NADPH formation monitored spectrophotometrically at 340 nm. H_2O_2 generation was measured by the Amplex UltraRed fluorescent assay.

3.3. *In vivo* measurements

A treadmill fatigue test was performed after two days of training. On the third day, mice were required to run on a treadmill with increasing speed; otherwise, the test was terminated. Echocardiography was performed on isoflurane-anesthetized animals. Cognitive tests were also conducted. In the open-field test, the apparatus consisted of a circular arena subdivided into multiple sectors. Locomotor activity and exploratory drive were quantified by the number of line crossings and the frequency of rearing events. The Morris water maze test was conducted in a circular, black-walled tank with a circular escape platform submerged at a fixed location below the water surface. Animals were tested over five consecutive days, with one session per day, each comprising four trials initiated from different starting positions around the perimeter of the tank in varying order. Within each daily session, the latency in the first trial was used as a measure of reference memory, while the mean latency across all four trials served as an index of working memory.

3.4. Histological analysis

For histological studies, samples were prepared using a routine formalin-fixed, paraffin-embedded process. Skeletal muscle fibrosis was assessed by Sirius Red staining. Immunohistological analyses of brain samples were carried out using specific antibodies: anti-CD68 for activated microglia; terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) for cell death; anti- dynamin-related protein 1 (Drp1) for mitochondrial fission; anti- mitofusin 2 (Mfn2) for mitochondrial fusion; anti- peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α) and anti-nuclear factor (erythroid-derived 2)-like 2 (Nrf2) for mitochondrial redox homeostasis and biogenesis; and anti-mitochondrial transcription factor A (TFAM) for mitochondrial DNA synthesis.

4. Results

4.1 *In vitro* measurements

4.1.1. Oxygen consumption and ATP production

Both oxygen consumption and ATP production decreased in DKO mitochondria of both ages when α-KG was used as the respiratory substrate. As a control, succinate-linked respiration and ATP production were also measured; no differences were observed between genotypes or age groups.

4.1.2. ROS production

In the presence of α-KG and the Complex I inhibitor rotenone, high rate of ROS generation was measured in all experimental groups due to elevated NADH/NAD^+ ratio. Under this condition, ROS formation was significantly lower in all DKO groups compared to WT groups.

The ROS generation was also measured using succinate as a respiratory substrate under conditions favoring reverse electron transfer (RET). Under these conditions, a large amount of ROS

was generated in WT animals, whereas DKO mice produced significantly less ROS. Moreover, aconitase activity, which is highly sensitive to ROS formation, was maintained in DKO mice after 15 minutes of succinate incubation. This result further strengthens the fact that the DKO mitochondria produce less ROS compared to the control.

4.2 *In vivo* measurements

4.2.1. Treadmill fatigue test

The DKO animals metabolically compensate well *in vivo* for a heterozygous double-knockout affecting the TCA cycle under resting circumstances. The question arose as to how the DKO mice behave under stress situation. The treadmill fatigue test was performed until full exhaustion on a motor-driven treadmill with increasing velocity. During the active phase (nocturnal period), DKO200 mice exhibited reductions in total running distance and time, relative to age-matched controls. Meanwhile, there were rather a trend in the younger animals. These findings suggest that under conditions of KGDHc deficiency, the elevated energetic demands imposed by intense physical exercise cannot be adequately met, probably owing to impaired mitochondrial ATP synthesis and reduced O_2 utilization.

We further hypothesized that fibrotic remodelling of skeletal muscle tissue may have contributed to the impaired exercise endurance observed in DKO mice. However, histological examination using Sirius red revealed no evidence of fibrotic changes in DKO animals.

4.2.2. Echocardiography

Cardiac dysfunction was considered as a potential contributing factor to the accelerated exhaustion observed in the forced treadmill test. Analysis revealed reductions in left ventricular end diastolic volume (LVEDV) and stroke volume (SV) in the DKO100 but not in the DKO200 animals, which were accompanied by a significant decrease in cardiac output (CO) both in DKO100 and DKO200 groups. Ejection fraction (EF)

and fractional shortening (FS), however, remained unaltered (the same is true for average heartbeat).

4.2.3. Open field test

KGDHc deficiency has been consistently associated with cognitive decline and impaired motor function in the literature. However, no behavioral assessments have been reported with KGDHc subunit-specific knockout animal models without administrating (neuro)toxic chemicals. Novelty-induced horizontal locomotor activity, as quantified by the number of line crossings, was significantly reduced both in DKO100 and DKO200 groups relative to controls. Vertical activity, assessed by the frequency of rearing events, also exhibited a declining trend in DKO groups compared to WT animals. Nevertheless, the time spent in the inner and in the outer zones of the arena did not differ between genotypes.

4.2.4. Morris water maze test

In this experiment, working memory was markedly impaired in DKO200 animals relative to controls, whereas differences in reference memory, although directionally consistent, remained only suggestive. In this test, there was no difference between DKO100 and WT100 groups.

4.3 Histological examination

4.3.1. Microgliosis and cell death markers

To determine the origin of the changes observed in behavioral tests, histological samples were taken from different parts of the brain. CD68 expressed predominantly in microglial cells within the brain, especially, in activated form. Microgliosis is an indicative of cortical neuronal damage arising from energy deprivation, which leads to the release of damage-associated molecular patterns (DAMPs) that subsequently trigger microglial activation. In the cerebral cortex of the DKO200 mice, substantial microgliosis was found through anti-$CD68^{+}$ immunostaining, pointing to neuroinflammation.

Immunohistology samples from the hippocampal region were prepared using TUNEL method. With this staining method, the terminal deoxynucleotidyl transferase specifically binds to the free 3'-OH end of DNA. In aged DKO animals, the CA1 region of the hippocampus exhibited extensive DNA fragmentation compared to age-matched controls.

4.3.2. Mitochondrial markers

In both neuronal and glial cells of DKO200 animals, the relative expression of Drp1, a key mediator of mitochondrial fission, was found to be elevated. In line with that, relative expression of Mfn2, which plays a central role in mitochondrial fusion, was significantly reduced in DKO200 mice. The expression level of MTFA remained unchanged in DKO200 group relative to WT200, indicating that the introduced combination of mutations does not alter or affect mitochondrial DNA integrity or replication. PGC-1α and Nrf2 were both found to be downregulated in neurons and glial cells of older DKO mice relative to older WT animals. Importantly, PGC-1α and Nrf2 are parts of the same redox signalling pathway controlled by p53. Consistent with previous reports, the reduction in PGC-1α expression in the context of mitochondrial dysfunction has been associated with inflammatory responses, neurite degeneration, and cell death.

5. Conclusions

Our study with DKO animals shows that significant substrate-specific alterations in mitochondrial bioenergetics *in vitro* led to only minor metabolic and behavioral differences between the two genotypes *in vivo* under physiological, resting condition. The major finding of our study is that the middle-aged transgenic mice demonstrated significantly poorer performance than the corresponding younger animals under high energy demand situations, like stress conditions. Under these circumstances, the

functional alleles of the *DLST* and *DLD* genes were ineligible for outweighing the altered ATP usage. In the fatigue-endurance treadmill test, middle-aged DKO animals reached exhaustion significantly earlier and covered a considerably shorter total running distance compared to their age-matched controls. This impairment is attributable not only to the underlying energetic deficit, but also to the mildly reduced resting cardiac function, which was further exacerbated upon physical exertion. Furthermore, heterozygous knockout of both DLST and DLD was found to induce microgliosis in the cerebral cortex, neuronal death in the hippocampus, and dysregulation of mitochondrial dynamics and biogenesis in middle-aged mice. Collectively, these findings indicate significant neuronal involvement and are consistent with a mild but discernible cognitive decline, which was further corroborated, at least in part, by the results of the behavioral test.

6. Bibliography of the candidate's publications

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