Genome instability in Maple Syrup Urine Disease correlates with impaired mitochondrial biogenesis

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ABSTRACT

Objective. The mitochondrial branched-chain ketoacid dehydrogenase (BCKD) catalyzes the degradation of branched-chain amino acids (BCAA), which have been shown to induce oxidative stress. Maple Syrup Urine Disease (MSUD) is caused by impaired activity of BCKD, suggesting that oxidative stress and resulting DNA damage could contribute to pathology. We evaluated the potential effect of BCKD deficiency on genome integrity and mitochondrial function as a downstream target.

Methods. Primary fibroblasts from MSUD patients and controls were either cultivated under normal conditions or exposed to metabolic or oxidative stress. DNA was analyzed for damage and mitochondrial function was evaluated by gene expression analyses, functional assays and immunofluorescent methods.

Results. Patient fibroblasts accumulated damage in mitochondrial DNA (mtDNA) and nuclear DNA, with a corresponding reduction in mitochondrial transcription, mtDNA copy number and pyruvate dehydrogenase. We found no evidence of increased level of reactive oxygen species (ROS) in patient fibroblasts under normal conditions, suggesting that the genotoxic effect is ascribed to accumulating metabolites.

Conclusions. Impaired BCKD activity as in MSUD, results in accumulation of DNA damage and corresponding mitochondrial dysfunction.

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1. Introduction

MSUD is an autosomal recessive disease, caused by mutations in one of the genes encoding subunits of BCKD. MSUD has been classified into five distinct forms depending on the severity of the disease, affected gene and responsiveness to thiamine treatment [1]. Mutations in the DBT gene coding for the transacetylase (E2) subunit are often associated with the intermittent form of the disease, where patients have some residual enzyme activity left [2]. Our laboratory previously identified new mutations in the E2 subunit in patients with the intermittent variant of MSUD and showed that the...
mutations lead to instability of the E2 protein with subsequent lower cellular level [3].

The BCKD complex catalyzes the rate-limiting step in the degradation of the three branched-chain amino acids (BCAAs), leucine, isoleucine and valine. During MSUD, BCAAs and their corresponding keto acids (BCKAs; α-ketoisocaproate, α-keto-β-methylvalerate and α-ketoisovalerate) accumulate, which can lead to encephalopathic crisis, mental retardation or death if left untreated. Neuropathology in MSUD, which can lead to encephalopathic crisis, mental retardation includes dysmyelination [4,5] is reported to correlate with DNA damage introduced into a Taq1 sensitive restriction site will result in altered cutting frequency of the DNA, which ultimately will affect PCR amplification of the target sequence spanning the restriction site. The resulting Δct gives an estimate of the DNA damage level. The method has been developed by us previously [20]. mtDNA copy number was estimated by real-time qPCR quantification of the mtDNA-encoded mt-RNR1 relative to the nuclear NDUFA9 using the ΔΔct method and presented relative to average of controls.

2.4. Gene expression

Gene expression analysis was performed by qPCR on StepOne Plus Real-time PCR system (Applied Biosystems/Life Technologies) using Power SYBR green master mix (Applied Biosystems/Life Technologies) and primers listed in Table 1. cDNA was synthesized from total RNA with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Expression levels were determined with the relative standard curve method, and the housekeeping gene 18S rRNA was used as internal control.

2.5. ROS Analysis, ATP measurement and citrate synthase activity

Mitochondrial ROS generation in vivo was quantified after staining plated fibroblasts with 100 nmol/L rhosamine (MitoTracker® Red CMXRos, 1 mmol/L stock solution in DMSO, Invitrogen/Molecular Probes, Eugene, OR) and monitoring for 30 min. The cells were assessed by confocal imaging at regular intervals up to 30 min, with a 40× oil objective on a Leica TCS SP8 as described below. Intensity histograms were collected for each time point for two cells in eight different locations from four patients and four controls, and mitochondrial ROS

<table>
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<th>Target</th>
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2.2. Nucleic acid isolation and quantification

Total DNA was isolated from cells with Blood and Tissue kit (Qiagen) according to manufacturer’s protocol. Total RNA was isolated with RNasy mini kit (Qiagen) according to manufacturer’s protocol, including an optional DNase treatment step. Nucleic acid concentrations were estimated by Nanodrop Spectrophotometer (Thermo Scientific), or alternatively by Epoch Microplate Spectrophotometer (Bio-Tek).

2.3. DNA damage and copy number estimation

DNA damage was estimated by a method based on the ability of DNA damage to inhibit restriction enzyme cleavage. DNA damage introduced into a Taq1 sensitive restriction site will result in altered cutting frequency of the DNA, which ultimately will affect PCR amplification of the target sequence spanning the restriction site. The resulting Δct gives an estimate of the DNA damage level. The method has been developed by us previously [20]. mtDNA copy number was estimated by real-time qPCR quantification of the mtDNA-encoded mt-RNR1 relative to the nuclear NDUFA9 using the ΔΔct method and presented relative to average of controls.

2.1. Cell material, culture and treatment

This study was approved by the Regional Committee for Medical and Health Research Ethics of South East Norway, and the skin fibroblasts for culture were collected and stored in approved biobanks according to Norwegian framework. Primary fibroblasts from four intermittent MSUD patients and four healthy controls were used. The patients carrying the E2 amino acid alterations R301C, G62X, W84C and R376C, respectively, have previously been described [3]. The fibroblasts were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mmol/L glutamine and 1% Pencillin/Streptomycin supplement. During treatment, the fibroblasts were exposed to 10 mmol/L BCAA (Sigma Aldrich) for 24 h before the cells were harvested and analyzed.

Table 1 – Oligonucleotides used in this study.

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3.1. DNA damage accumulates in MSUD fibroblasts

The primary fibroblasts from four MSUD patients that were used in this study have been characterized previously [3]. All four patients are carriers for the E2 R301C mutation and an additional allelic mutation that fail to produce a stable protein. The remaining R301C mutant protein constitutes about 20% of normal cellular level of the E2 (Supplementary Fig. S1) and a corresponding lower catalytic activity [3]. BCKD-E2 is identified in nucleoid screens from phylogenetically distinct mitochondria [17,19], inferring that the E2 protein has a separate, evolutionary conserved role in this mitochondrial chromatin. Since the assembly of the nucleoid depends on its factors [21], we reasoned that E2 depletion as seen in the patient fibroblasts might influence the mtDNA. In fact, when we assessed mtDNA damage in patient fibroblasts, we found a significantly higher level of damage in the mtDNA compared to mtDNA from healthy controls (Fig. 1A). To elaborate on the putative protective role of E2, we induced oxidative stress by exposing cells to menadione. The patient cells accumulated mtDNA damage to the same extent as control cells upon this treatment (Supplementary Fig. 2A), and therefore do not support a general mtDNA-protecting role of E2. As the BCKD represents the rate-limiting step in the catabolism of BCAA (and BCKA), we investigated further the mtDNA damaging effect of these metabolites. mtDNA damage was induced in control fibroblasts upon exposure to BCKAs, but not BCAA. The damage load was comparable to that observed in non-treated patient fibroblasts (Fig. 1B and A).

We further investigated the integrity of the nuclear DNA. Using the same approach targeted to the NDUFA9 gene encoding one of the subunits of complex I, we found significantly more nuclear DNA damage in patient fibroblasts, implying that depletion of E2 induced a general genotoxic stress in these cells, presumably due to accumulation of BCKAs or alternatively other metabolites accumulating during BCKD insufficiency (Fig. 1C).

To confirm that the patient fibroblasts display a different metabolite profile than control cells, we assessed the intracellular acylcarnitine levels in cell free extracts. Catabolism of BCKA via decarboxylation by BCKD yields the CoA intermediates isovaleryl-CoA, β-methylbutyryl-CoA (C5), isobutyryl-CoA (C4) and CoA derivatives acetyl-CoA, propionyl-CoA (C3) and succinyl-CoA (C4) [22]. Correspondingly, the C3 and C4 acylcarnitines were reduced 8- and 5-fold, respectively in these cells (Supplementary Fig. S3). The small reduction in C4 acylcarnitines in patients was not statistically significant. In addition to BCKA, metabolites from other BCKD requiring processes have potential DNA damaging effect. The methionine salvage pathway includes the BCKD complex [23] and we tested two of the degradation metabolites; 4-methylthio-2-oxobutanoate (MTOB) and methional for their ability to induce mtDNA damage. While MTOB was found to be harmless to mtDNA damage, methional exposure led to substantial decrease in the mtDNA integrity (Supplementary Fig. S2B). In conclusion, these data are indicative of another metabolic profile in patient fibroblasts, which has genotoxic effects.

The similar response to menadione in patient and control fibroblasts implies that the ROS defense is similar in the two cells. In order to address this directly, we assessed generation of mitochondrial ROS. Despite the elevated (mt)DNA damage level in patient fibroblasts, the generation of mitochondrial ROS was significantly lower (Fig. 2). The increased mitochondrial ROS was supported by reduced peroxide emission from the patient fibroblasts, which was not significantly affected by bolus doses of BCKA (Supplementary Fig. S4). Thus, we
conclude that ROS is dispensable for the elevated DNA damage in patient fibroblasts.

As an attempt to address the specific role of E2 subunit in protecting cellular DNA, we used siRNA to compare the effects of depleting BCKDA, encoding the E1α subunit of BCKD, and DBT (encoding the E2 subunit) on the DNA integrity in the bone osterosarcoma cell line U2OS. The E1α subunit harbors the decarboxylase activity of BCKD complex, whose activity is regulated by phosphorylation of the Ser293 residue. Transient transfection of siRNA resulted in 20 and <10 percent of normal levels of DBT and BCKDA, respectively (Supplementary Fig. 5A). Interestingly, reduction of DBT had a genotoxic effect, as inferred from the increased damage in both nuclear and mitochondrial DNA (Supplementary Fig. 5B). In contrast, depleting BCKDA levels had no significant effect on DNA integrity. These data suggest that E2 has a specific role distinct from the BCKD activity in preventing DNA damage.

3.2. Mitochondrial dysfunction in MSUD fibroblasts

Persistent mtDNA damage has been shown to induce mtDNA degradation and reduce expression of mtDNA genes in addition to causing reduced mitochondrial function [24]. In line with this, mtDNA copy number and expression of mtDNA genes were reduced approximately 30% in the patient cells (Fig. 3A). The expression of MTRNR1 encoding the 12S ribosomal RNA and MTND6 from the mtDNA was significantly reduced in patient fibroblasts, while MTND2 levels were normal (Fig. 3B). For comparison, the expression of the nuclear encoded ATP5A1 and NDUFA9 mitochondrial genes was not affected. The reduction of mtDNA correlated with a 20% reduction in the cellular citrate synthase activity, which is an established marker for mitochondrial mass (Fig. 3C). The reduction in mitochondrial parameters was manifested as a tendency to lower respiration capacity (Supplementary Fig. S6) and lower cellular ATP (Fig. 3D).

3.3. Metabolic alterations in MSUD fibroblasts

The NAD/NADH ratio describes the redox state of the cell and depends on and regulates the mitochondrial activity and cell homeostasis [25,26]. The NAD/NADH ratio was reduced in patient fibroblasts (Supplementary Fig. S7A), compared to control cells. Apart from being an important redox molecule, NAD+ is an essential co-factor in many enzymatic reactions like those catalyzed by the sirtuins (Silent information regulator of gene transcription-family). To investigate the effect of the reduced NAD/NADH ratio, we evaluated the expression levels of four different sirtuins; SIRT1 which responds to mtDNA damage [27] and SIRT3-5 which are located in mitochondria [28]. Expression of SIRT4 is significantly reduced in the patient fibroblasts, while the other sirtuins were normally expressed (Supplementary Fig. S7B). SIRT4 is in contrast to SIRT1-3 not a NAD-dependent deacetylase, but it inhibits the glutamate dehydrogenase.
(GDH) by ADP-ribosylation [29]. When adjusted for the reduced mitochondrial volume, the mitochondrial activity of GDH was increased (Supplementary Fig. S7C), which is in correlation with reduced SIRT4 activity.

BCKD has similar structure to α-ketoglutarate dehydrogenase and pyruvate dehydrogenase (PDH). Like BCKD, the PDH complex is assembled around a core of its E2 subunits and one study has identified PDH-E2 in the mitochondrial nucleoid [17]. PDH localizes to a distinct subpopulation of mitochondria [30] and in order to evaluate potential heterologous distribution of BCKD, we analyzed the intracellular localization of PDH and BCKD by immunofluorescence with antibodies against the respective E2 subunits. Interestingly, the BCKD-E2 and PDH-E2 were distinctly distributed throughout the mitochondrial network. The complexes were identified in some common loci, whereas the majority of each complex was residing in separate BCKD-E2 or PDH-E2-specific mitochondrial compartments (Fig. 4A). It was therefore a possibility that the depletion of E2 in patients selectively reduced the BCKD-containing fraction of cellular mitochondria. We addressed this by analyzing the localization of PDH. There was an overall 30% reduction in PDH signal from patients that correlated with lower gene expression (Fig. 4B and C). Thus, these results show that BCKD insufficiency somehow influences the cellular level of PDH in these primary fibroblasts. When we compared the cellular level of PDH in the two cell types, it was evident that the PDH-enriched mitochondria were relatively sparse in the patient fibroblasts (Fig. 4D).

4. Discussion

MSUD is an autosomal recessive metabolic disorder characterized by ketoacidosis and neurological dysfunction, whose underlying mechanisms are less understood. In this study, we have investigated the DNA integrity and mitochondrial function in fibroblasts from intermittent MSUD patients. We demonstrate that BCKD-E2 insufficiency as in intermittent form of MSUD results in accumulation of DNA damage and a corresponding reduction in mitochondrial markers. mtDNA integrity is essential for normal cellular activity and function [24,31,32]. The increased DNA damage level we find can be mimicked in healthy control fibroblasts through the administration of BCKA. This indicates that DNA damage caused by BCKA is one of the causative factors underlying the pathophysiology in MSUD. In a study by Scaini and coworkers, they report similar findings in hippocampus and striatum in the brain after acute and chronic administration of BCAAs in rats [14]. They did not test the effect of BCKA administration. Working with an in vivo model, they cannot account for the contribution to DNA damage from the BCKAs. In brain, there is high aminotransferase activity that will rapidly convert...
BCAAs to their corresponding keto acids. The doses we used to induce DNA damage are quite high although they are commonly used to induce MSUD-like effects in vitro [33]. Normal plasma concentrations of BCAAs and BCKAs are around 0.1–0.2 mmol/L, but can increase 50-fold during metabolic crisis [34]. We previously reported 2.2 mmol/L serum concentration of leucine in patients, which correlated with 86% loss of decarboxylase activity in the patient fibroblasts [3], yet, the intracellular concentration may vary in a cell- and condition dependent manner.

ROS is a side effect of aerobic metabolism and the reduction in ROS is likely to be a manifestation of reduced mitochondrial capacity. More specifically, PDH is capable of producing significant amounts of ROS in the form of superoxide anion [35]. The reduction in mitochondrial ROS found here may therefore at least partly be explained by the lower PDH levels in patient fibroblasts.

As BCKD-E2 has been identified as an mtDNA-associated factor in the nucleoid, we wondered if the E2 depletion might render mtDNA vulnerable to damage. However, the patient fibroblasts were as sensitive as control cells to the exogenous oxidant menadione. Also, nuclear DNA was more affected in the patient fibroblasts compared to control cells. Interestingly, when we compared the effects of diminishing E1α and E2 subunits in the U2OS cell line, we found that E1α was dispensable for the DNA protective effect. Knock down of BCKD-E2 has been identified as an mtDNA-associated factor in the nucleoid, we wondered if the E2 depletion might render mtDNA vulnerable to damage. However, the patient fibroblasts were as sensitive as control cells to the exogenous oxidant menadione. Also, nuclear DNA was more affected in the patient fibroblasts compared to control cells. Interestingly, when we compared the effects of diminishing E1α and E2 subunits in the U2OS cell line, we found that E1α was dispensable for the DNA protective effect. Knock down of BCKD-E2 led to a compensatory increase in DBT that potentially could have a DNA-protective effect (Supplementary Fig. S5).

Thus, these observations infer that E2 is relatively more important for genomic stability than E1α. The distinct effects of depleting two subunits of the same complex may appear counter intuitive, but could potentially result from an unbalanced decarboxylase activity of E1α relative to the transacylase activity of E2. This could result in leakage of reactive, genotoxic metabolites, such as methional from the BCKD complex ([23] and Supplementary Fig S2B). BCKA induced damage in both cellular genomes. Although BCKD is a mitochondrial enzyme and this could explain the sensitivity of mtDNA to accumulated BCKAs, BCAT, the enzyme responsible for the transamination of BCAA to BCKA is located in both the mitochondria and the cytosol [36]. Thus it can be expected that BCKA is distributed throughout the cell and affects nuclear DNA to a similar extent as mtDNA.

**Fig. 4** – E2 subunits of PDH and BCKD are located in distinct mitochondria, and both are reduced in MSUD patient fibroblasts. (A) Heterologous cellular distribution of PDH-E2 and BCKD-E2. Intracellular localization of PDH-E2 (red) and BCKD-E2 (green) was assessed by confocal imaging as described. Nucleus is visualized by DRAQ5 (blue). (B) Expression of PDH-E2 mRNA was analyzed by RT-qPCR, normalized to 18S rRNA and presented relative to the mean of controls, with SE. (C) Representative image of PDH-E2 in patient and control cells. (D) The cellular content of loci with a specific PDH-E2 level in patient fibroblasts was related to that in control fibroblasts and plotted against PDH-E2 intensity (pixel intensity was obtained by identical instrument settings). Average ratios from four controls/patients, each based on 15 different cells were determined by ImageJ software. The red bar illustrates the range of PDH-E2 intensities that are significantly underrepresented in patients compared to control fibroblasts. *p < 0.05.
Based on the synchronized reduction of citrate synthase, mtDNA copy number, MTND6 expression, PDH level and ROS generation in the patient fi broblasts, we conclude that impaired mitochondrial biogenesis is implicated in MSUD. The closest cause of consequence appears to be the increased mtDNA damage, which has been shown to reduce miRNA transcript levels in other systems [20,37]. Notably, we found that expression of MTND2 from the heavy strand promoter is not comparably reduced. It has been shown that biased accumulation of mutations in the two promoter regions can selectively alter replication and transcription from the light strand promoter [38], however, we have no evidence for a regionally accumulation of damage in this region in mtDNA in patient fi broblasts.

The cellular consequences of deficiency in mitochondrial function include a variety of direct and secondary changes in metabolite homeostasis, such as NAD/NADH ratio and ATP level. MSUD patient fi broblasts have a lower NAD/NADH ratio than control cells. Although this ratio is affected by several different activities within the cell, it can be generally considered to refl ect the metabolic state and the health of a cell [39].

In summary, our results indicate that accumulation of metabolites due to defective BC KD-E2 causes DNA damage which leads to lower mtDNA copy number, reduced mtDNA transcript levels and compromised mitochondrial function in MSUD. The translational potential in our fi ndings lies in addressing mitochondrial parameters in the treatment and disease outbreak in MSUD patients. The strength of the correlation between E2 function and DNA damage may have the potential to be used in therapy.

Author contributions

JS; RS; KS designed and performed the experiments, analyzed data and wrote the manuscript, BW, TR and MB analyzed data and wrote the manuscript, LE designed experiment, analyzed data and wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.metabol.2014.05.003.

REFERENCES


