Mitochondrial and Gene Expression Changes in Brain Tumor Cells Grown in β-Hydroxybutyrate

Abstract # TMET-24

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Abstract

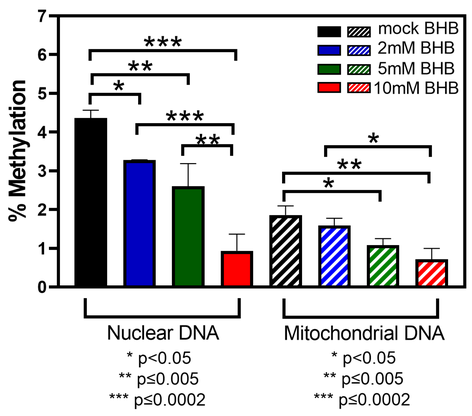
Malignant brain tumors are devastating diseases with poor outcomes. The search for more effective therapeutic combinations has led to investigation of the use of a therapeutic ketogenic diet (KD) as an adjuvant therapy based on the metabolic dysregulation that is a hallmark of cancer [1]. KD sometimes results in extended survival, but like all cancer therapies the tumor eventually becomes resistant. Resistance can arise due to the "metabolic remodeling" of resistant cells due to limitations in nutrient availability and other signals from the tumor microenvironment. We are investigating the molecular and mitochondrial changes of tumor cells grown in β-hydroxybutyrate (BHB), the main ketone produced when following a therapeutic KD, to partially recapitulate the long-term effects of the KD on patients.

Murine malignant glioma cells (GL261-luc2) were cultured long term in 2, 5 or 10mM BHB. The Agilent XFe96 Seahorse Analyzer was used to measure changes in cell metabolism. Epigenetic changes that occur in the mitochondria were interrogated using DNA methylation assays. Finally, we implanted BHB resistant glioma cells intracranially in mice and carried out RNAseq to determine the mechanisms by which these cells adapt.

Seahorse analyses of GL261-luc2 cells grown in BHB demonstrated changes in several respiratory parameters including maximal respiration and reserve capacity. In addition, there is a statistically significant decrease in mitochondrial DNA methylation with increasing concentrations of BHB. RNAseq analysis of BHB resistant GL261 tumors showed changes in pathways related to mitochondrial translation and the electron transport chain.

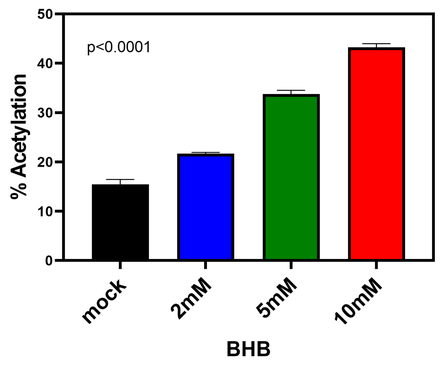
Our preliminary results show that the heterogeneity seen in these tumors is recapitulated in the various mitochondrial respiration parameters. Further, these may be altered as a consequence of BHB therapy. An understanding of the genetic and mitochondrial changes that occur when cells become resistant will lead to a more effective use of the KD as an adjuvant therapy.

BHB Reduces DNA Methylation



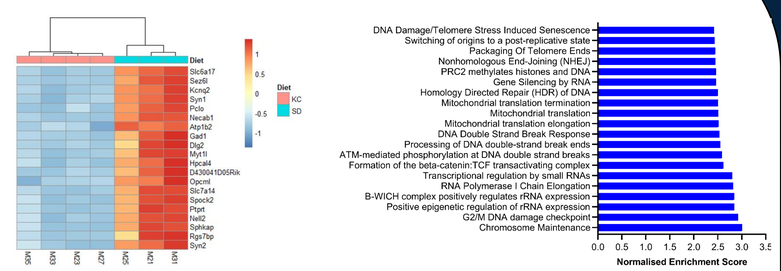
There is a statistically significant decrease in mitochondrial and nuclear DNA methylation in cell lines selected for growth in increasing concentrations of BHB.

BHB Increases Histone H3 Acetylation



There is a statistically significant increase in histone H3 acetylation in cell lines selected for growth in increasing concentrations of BHB.

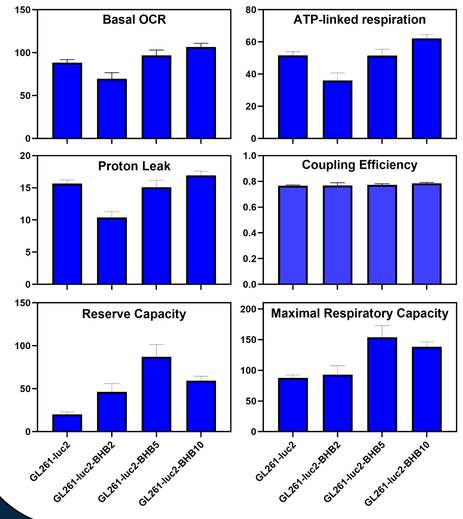
Gene Expression Differs in GL261-BHB^{res} Tumors From Mice Fed a SD vs KD

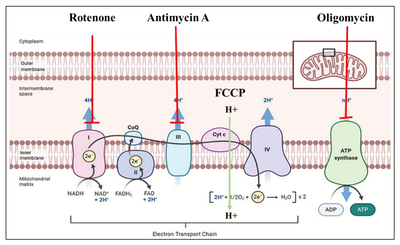


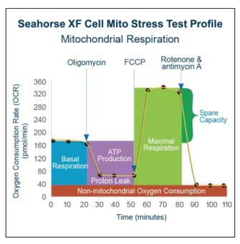
Heatmap showing the top 20 differentially expressed genes between mice fed a standard diet (SD) vs a ketogenic diet (KC, KetoCal®). RNAseq data from GL261-BHB^{res} tumours were analysed using DESeq2. Differential gene expression was measured using APELGM method. Heatmap generated using the pheatmap package for the 20 genes with the lowest p values. Clear separation of the SD (n = 3) and KD (n = 4) treated samples can be seen for these genes.

Bar chart showing the top altered molecular pathways between SD and KD. RNAseq data from GL261-BHB^{res} tumours were analysed using DESeq2. Differential gene expression was measured using APELGM method, and log-rank scores were generated for all genes. Scores and ENTREZ gene identifiers were passed to WebGestalt for Gene Set Enrichment Analysis (GSEA). GSEA was carried out for 16078 unique genes in Reactome pathways. Analysis showed major changes in pathways related to mitochondrial translation and DNA repair.

Respiratory Parameters of GL261-luc2 Cells Grown in BHB

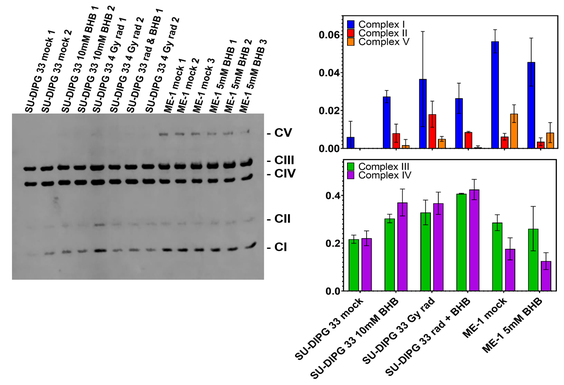






Seahorse analysis of GL261-luc2 cells grown long-term in BHB show changes in the mitochondrial reserve capacity and maximal respiratory capacity compared to that of the untreated GL261-luc2 cells.

Western Blot Analysis of OxPhos Mitochondrial Proteins



The DIPG cell line SU-DIPG33 was compared to normal fibroblasts (ME-1) with and without a 4-day treatment with 10mM BHB and 4Gy radiation after the first day of BHB treatment. There is an increase in complexes III and IV as well as a striking loss of complex V in the tumor cells compared to the normal fibroblasts (ME-1).

Methods

Cell Lines: GL261-luc2 murine glioma cells were cultured and maintained as described [2]. ME-1 cells were cultured and maintained under the same conditions using 20% FCS. SU-DIPG 33 cells were a generous gift from Dr. Michelle Monje (Stanford University) and were cultured as described [3].

Beta-hydroxybutyrate (BHB): GL261-luc2 cells were grown long term in freshly made media containing 2, 5 or 10mM (R)-(−)-3-hydroxybutyric acid sodium salt (Sigma-Aldrich, St. Louis, MO). SU-DIPG 33 cells were treated for 4 days prior to protein isolation for western blot analysis.

Western Blot Analysis: Protein isolation and Western blot analysis was done as described [2] using a total OXPHOS human WB antibody cocktail (ab110411, abcam, Waltham, MA) using conditions specified by the manufacturer. Quantitation was done using REVERT™ Total Protein Stain (Li-Cor Biosciences, Lincoln, NE) as a loading control, images were captured on the Li-Cor Odyssey® Fc near-infrared imager and analysis was done using Image Studio (Li-Cor Biosciences).

DNA Methylation: GL261-luc2 cells cultured with and without BHB were collected and mitochondrial DNA isolation was done using a Mitochondrial DNA Isolation Kit (ab65321, abcam) with conditions specified by the manufacturer. Mitochondrial and total DNA methylation was determined using a Global DNA Methylation Assay Kit (ab233486, abcam) using conditions specified by the manufacturer.

Histone H3 Acetylation: GL261-luc2 cells cultured with and without BHB were collected and global histone H3 acetylation was analyzed using the Histone H3 Acetylation Assay Kit (ab115102, abcam) using conditions specified by the manufacturer.

Metabolic Analysis: Respiratory activity of GL261 cells was studied using the Agilent XFe96 Seahorse Analyzer and Seahorse XFp Cell Mito Stress Test Kit (Agilent, Santa Clara, CA) using conditions specified by the manufacturer.

In vivo analysis of GL261-luc2-BHB^{res} Cells: BHB resistant GL261-luc2 glioma cells were implanted intracranially in mice as described [4,5]. Mice were maintained on a standard diet (SD) or ketogenic diet (KD; KetoCal® 4:1, Nutricia North America, Gaithersburg, MD) [4,5] and RNAseq was done on the resulting tumor tissue.

Conclusions

- The ketone BHB causes changes in various mitochondrial parameters, *even in the absence of glucose restriction.*
- Long term treatment of murine malignant glioma cells causes dose-dependent epigenetic changes including a significant reduction of both nuclear and mitochondrial DNA methylation and an increase in histone H3 acetylation. These epigenetic changes may explain, in part, the far-reaching anti-tumor effects of ketones when used alone and as an adjuvant therapy.
- Long term treatment of murine malignant glioma cells alters mitochondrial function, specifically maximal respiratory capacity and reserve capacity.
- Differential expression of the mitochondrial OXPHOS complexes can occur in response to BHB treatment in cells from human malignant tumors such as SU-DIPG 33.
- Intracranial syngeneic tumors from BHB resistant cells show differential gene expression profiles in mice fed a standard diet vs a ketogenic diet. Specifically, major changes can be seen in pathways related to mitochondrial translation and DNA repair.
- A greater understanding of changes that occur in the tumors of patients on long-term ketogenic therapy may suggest additional adjuvant treatment(s) to enhance survival.

References

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