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Temperature induces significant changes in both glycolytic reserve and mitochondrial spare respiratory capacity in colorectal cancer cell lines

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ABSTRACT

Thermotherapy, as a method of treating cancer, has recently attracted considerable attention from basic and clinical investigators. A number of studies and clinical trials have shown that thermotherapy can be successfully used as a therapeutic approach for various cancers. However, the effects of temperature on cancer bioenergetics have not been studied in detail with a real time, microplate based, label-free detection approach.

This study investigates how changes in temperature affect the bioenergetics characteristics (mitochondrial function and glycolysis) of three colorectal cancer (CRC) cell lines utilizing the Seahorse XF96 technology. Experiments were performed at 32 °C, 37 °C and 42 °C using assay medium conditions and equipment settings adjusted to produce equal oxygen and pH levels ubiquitously at the beginning of all experiments. The results suggest that temperature significantly changes multiple components of glycolytic and mitochondrial function of all cell lines tested. Under hypothermia conditions (32 °C), the extracellular acidification rates (ECAR) of CRC cells were significantly lower compared to the same basal ECAR levels measured at 37 °C. Mitochondrial stress test for SW480 cells at 37 °C vs 42 °C demonstrated increased proton leak while all other OCR components remained unchanged (similar results were detected also for the patient-derived xenograft cells Pt.93). Interestingly, the FCCP dose response at 37 °C vs 42 °C show significant shifts in profiles, suggesting that single dose FCCP experiments might not be sufficient to characterize the mitochondrial metabolic potential when comparing groups, conditions or treatments.

These findings provide valuable insights for the metabolic and bioenergetic changes of CRC cells under hypo- and hyperthermia conditions that could potentially lead to development of better targeted and personalized strategies for patients undergoing combined thermotherapy with chemotherapy.

1. Introduction

Thermotherapy (hyper- and hypothermia) has been utilized for the treatment of various diseases since ancient times. One of the oldest reports describing the use of hyperthermia for cancer dates to about 3000 BCE and is found in the Egyptian Edwin Smith surgical papyrus [1]. Despite centuries of research on thermotherapy, the mechanism by which this modality produces its clinical effectiveness noted in multiple randomized clinical trials across a spectrum of malignancies is yet unknown [2]. Currently, one of the main theories in the field of temperature treatment for malignancy is that exposure to hyperthermia leads to disruption of the mitotic cycle (G1 arrest) and apoptosis. Most experimental evidence underlying this theory is from *in vitro* studies in which cell cultures were exposed to prolonged periods (24–72 h) of hyperthermia (defined as exposure to 39–42 °C). In a recent study, Zhu et al. [3] reported that various cancer cells maintained at 39 °C for 72 h demonstrated mild inhibition of cell growth by arresting the cells at the G1 phase of the cell cycle which also resulted in hyperthermia-enhanced efficacy of several chemotherapeutic agents [3]. In contrast to the *in vitro* experiments, the clinical *in vivo* protocols typically utilize only short-term exposure to hyperthermia. For example, several human breast cancer protocols incorporate

Abbreviations: ATP, Adenosine triphosphate; AUC, Area under the curve; CRC, Colorectal cancer; DOX, Doxorubicin; ECAR, Extracellular acidification rate; FCCP, Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone; OCR, Oxygen consumption rate; OXPHOS, Oxidative phosphorylation; PDX, Patient-derived xenograft; SCID, Severe combined immunodeficiency; SRC, Spare respiratory capacity

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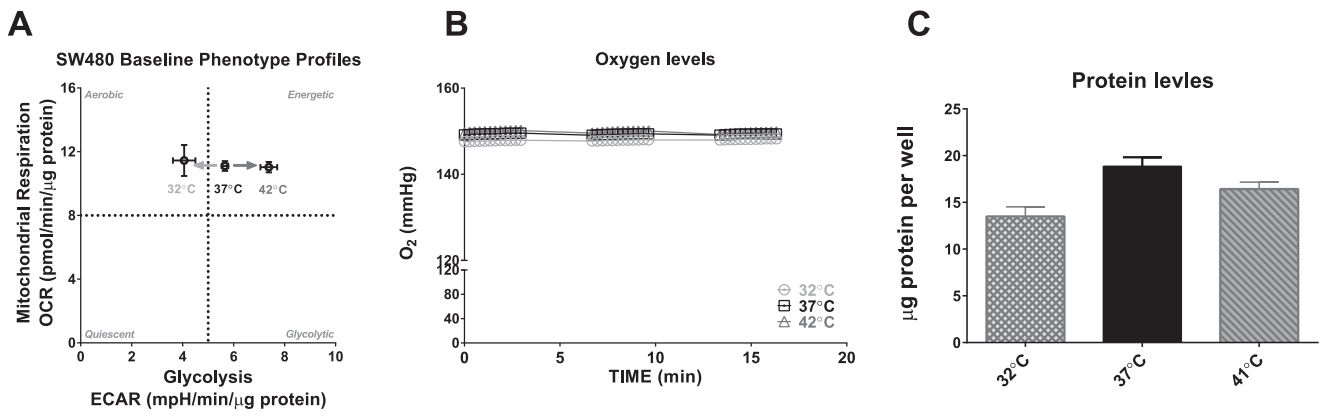

Fig. 1. Baseline energy profiles and oxygen levels. (A) OCR and ECAR levels of SW480 cell line incubated for approximately 60 min in XF modified media containing 25 mM glucose and 1 mM pyruvate. No statistical differences were found between OCR normalized to protein levels for the three different temperature conditions. The normalized to protein ECAR levels were significantly different for the 37 °C vs. 32 °C ($P=0.0093$, one-way ANOVA, Holm-Sidak's multiple comparisons test, n=14–16) and for the 37 °C vs. 42 °C conditions ($P < 0.0001$, one-way ANOVA, Holm-Sidak's multiple comparisons test, n=14–16); **(B)** Oxygen levels (mmHg) at the base-line time were not statistically different between temperature conditions. Oxygen levels shown represent control wells containing media alone without cells; **(C)** Protein levels were determined at the end of the experiments with BCA assay (n=8–16). One-way ANOVA with Bonferroni's multiple comparisons test show no significant differences between 37 °C and 41 °C ($P=0.2828$) and decreased in protein levels for the 32 °C group compared to 37 °C ($P=0.0020$).

radiation therapy hyperthermia treatments (41–42 °C) for ~60 min with at least 72 h between individual treatment sessions [4–6].

Since the first cancer-related Seahorse XF paper [7] was published in 2007, this technology has been exponentially utilized to study cancer cell metabolism. Currently, there are more than 500 cancer related-publications on various topics but few of them focus on the use of thermotherapy to modify cancer cell bioenergetics, metabolism and drug resistance. There is only one previous study using Seahorse technology to investigate effects of hyperthermia on cancer cell bioenergetics, which is focused only on basal levels of OCR. Curley et al. [8] used human pancreatic carcinoma cells to investigate how short term hyperthermia (cell heated up to 46 °C for 5 min) affects the base line oxygen consumption rates (OCR) when mitochondrial function is assessed 24 h post hyperthermia treatment on a Seahorse XF Extracellular Flux Analyzer [8]. The results suggest that the 5 min hyperthermia treatment reduced OCR by approximately 50%. Total OCR levels reported represent a sum of both non-mitochondrial respiration and base level respiration and were not normalized to cell count or protein levels.

The goal of our study was to perform a detailed analysis of the changes in cancer cell bioenergetics (mitochondrial and glycolytic cellular functions and their components) that occur in real time when cells are incubated for 1 h at temperatures equivalent to those commonly used in clinical practice for hypo- or hyperthermia. We chose to use colorectal cancer (CRC) cells for this study because CRC have been previously reported to the final slope of the cell survival curve where the curve approximates a straight line (aka final slope or D_0) at 43 °C applied for 1.5 h and when hyperthermia is combined with mitomycin C and cis-dichlorodiammineplatinum(II) treatment [9].

The results from our study indicate that changes in temperature (shifting from 37 °C to 32 °C or 42 °C for period of 90–120 min) significantly alters glycolysis and, to a less extent, modifies various components of mitochondrial function. Similar findings have been reported for yeast studies, where analysis of the high temperature-induced glycolytic flux suggested (without the use of Seahorse technology) that hyperthermia leads to a rapid increase in glycolytic flux that is not accompanied by an increase in respiration [10]. Secondly, the study demonstrates that the Seahorse XF technology can be successfully utilized to measure changes in cellular bioenergetics in conditions different from normal physiological temperature of 37 °C which is the standard condition for most cancer studies. Present findings might be of significance not only for cancer research, but also for other areas of biomedical research such as immunology, stem cells research and many others where potentially temperature could be used to modify cellular bioenergetics.

In summary, this study provides important insights into the nature of cancer cell response to thermotherapy, such as changes in metabolic potentials which can be potentially translated and utilized for the development of better clinical thermotherapy protocols for cancer treatment.

2. Materials and methods

2.1. Instrumentation, protocols and settings

All experiments were performed with the Seahorse XF 96 and XF software version 1.4. This system allows instrument temperature settings to be lowered (31–32 °C) or increased (41–42 °C) for the standard duration of typical mitochondrial and glycolysis stress tests. The maximum number of experiments completed in one day was three with increasing temperature (the first experiment was performed at 32 °C, the second at 37 °C, and the final experiment at 42 °C). In a separate set of experiments, the order of temperature testing (from 42 ° to 37 °C) was reversed to determine whether the order had any impact on the observed results. All OCR and ECAR analyses were performed at least two times with a minimal of 6–8 technical replicates (up to 80 replicates in some cases) for each treatment or assay. All cells were incubated for 60 min in a non-CO_2 incubator before plate calibration was performed and mitochondria and glycolysis stress test experiments were initiated at corresponding temperature conditions.

2.2. Cell lines

The human colon CRC cell lines, SW480 and HCT116, were obtained from ATCC (Manassas, Virginia) and authenticated in February 2016 (Genetica DNA Laboratories, Cincinnati, OH). All cell lines were grown in DMEM media containing 10% fetal bovine serum (FBS), 25 mM Glucose and 1 mM Pyruvate. At the time of experimentation, cells were in a passage range of 15–20 and cell seeding was 30,000 cells per well.

Following appropriate approval by the Institutional Review Board (IRB) at the University of Kentucky, CRC tissues were collected after surgical resection and implanted into NSG™, NOD *scid* gamma mice (The Jacksons laboratory) to established the patient-derived xenograft (PDX). The resultant primary CRC cell line (Pt. 93) was established after three sequential generations in NSG mice and authenticated as a unique human cell line in February 2016 (Genetica DNA Laboratories, Cincinnati, OH). For all of the Seahorse experiments, Pt.93 cells were