University of Nevada, Reno

An Exploration of the Metabolic Profile of Mammary Ductal Carcinoma Cells upon Treatment with Docosahexaenoic Acid

A thesis submitted in partial fulfillment
of the requirements for the degree of

Bachelor of Science, Biochemistry and Molecular Biology and the Honors Program

by

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Abstract

In recent years, cancer has come to be thought of as a metabolic disease. The Warburg phenotype, characteristic of most cancer cells is postulated to confer survival advantages. Special attention has been paid to treatments that attenuate metabolic function in cancer. Docosahexaenoic Acid (DHA; 22:6; 6n-3) has been shown to depress mammary carcinoma survival in cell culture as well as animal models. DHA supplementation has been shown to diminish the bioenergetic profile of malignant cell lines in a dose dependent manner. In the current study, the location of DHA's impact on oxidative phosphorylation will be investigated. This research will help to discern a possible mechanism for DHA, thus allowing for a more targeted approach in the search for compounds which may synergistically interact with DHA to inhibit cancer cell growth and proliferation.

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Introduction

Breast cancer is the second highest cause for cancer-related mortality in the United States, and over 231,840 women are estimated to be diagnosed in 2015 [1]. Although it has been almost a century since Otto Warburg observed a decrease in mitochondrial respiration and oxidative phosphorylation in favor of a glycolytic phenotype in cancer, it is only recently that cancer is being considered as a metabolic disease [2]. Importantly, the functional state of the mitochondria plays a critical role in carcinogenic invasion and metastasis because it has been shown to help stabilize the Warburg phenotype [3]. Although the majority of cancers exhibit a Warburg phenotype, a small subset of aggressive cancers display a mitochondrial phenotype which is heavily reliant on mitochondrial oxidative phosphorylation for energy.

It has been shown that hypoxia inducible factor 1α (HIF-1α) is at least partially responsible for altering the metabolic switch observed in cancer. Under normal conditions, HIF-1α mediates that adaptation of cells to hypoxic condition. However, in many cancers, HIF -1α has been shown to be stabilized even under normoxic conditions [4]. Studies have shown a correlation between poor prognosis [5], tumor aggressiveness [6], and early relapse [7] with increased HIF activity.

The importance of mitochondrial function in stabilizing cancer phenotype has led to the study of metabolic inhibitors as potential cancer therapeutics. For example, the mitochondrial targeted drugs MitoQ and Mito-CP have been shown do decrease cancer proliferation in vitro by lowering cellular ATP levels [8]. Additionally, dietary polyunsaturated fatty acids, in particular docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), have been shown to have anticancer properties. Recently,

it was shown that dietary treatment with DHA attenuates the bioenergetics profile of tumors by altering HIF-1α activity [9]. DHA has also been used in combination with the glycolytic inhibitor 2-deoxyglucose (2DG) to synergistically trigger cancer cell death [10]. However, no study has yet been performed that characterizes the combined effects of MTDs and DHA on cellular metabolism. Additionally, both MTDs and DHA are known to decrease cellular oxidative phosphorylation, but the location and mechanism underlying this effect is not well characterized. The current study is designed to elucidate the site at which DHA affects cellular respiration. Oxidative phosphorylation is carried out by a series of four protein complexes (I-IV) that are located in the inner mitochondrial membrane. A clark-type electrode will be employed to measure oxygen consumption in the presence and absence of specific mitochondrial inhibitors and substrates in order to determine specific activity and location of the DHA effect within each of the four complexes. In order to test this hypothesis, two metabolically distinct breast cancer cell lines will be employed. (1) BT-474, with a mitochondrially active phenotype, and (2) MDA-MB-231, with a glycolytically active (Warburg) phenotype. A non-transformed breast epithelial MCF-10A cell line will be used as a control. Together the data should help advance our current understanding of the root cause of DHA induced cancer bioenergetic reprogramming as well as provide evidence as to the possible enhancement of current therapies in the treatment of cancer.

Methods

Cell Lines & Reagents

BT-474 mammary ductal carcinoma, MDA-MB-231 adenocarcinoma, and MCF-10 non-transformed cells were purchased from ATCC (Manassas, VA). Docosahexaenoic acid (DHA; C22:6n-3) was purchased from Sigma (Sigma, St. Louis, MO) and dissolved in ethanol (EtOH), flushed with nitrogen gas, protected from light and stored at -20°C for no more than 60 days.

Cell Culture

BT-474 cells were maintained in HybriCare (ATCC) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT). MDA-MB-231 cells were maintained in RPMI-1640 (Thermo Scientific, Rochester, NY) supplemented with 10% FBS (Hyclone, Logan, UT). MCF-10A cells were maintained in DMEM/F12 (Thermo Scientific, Rochester, NY) supplemented with 5% FBS (Hyclone). Cells were grown as monolayers at 378C in a humidified environment with 5% CO_2.

Aerobic Metabolism Assay

The partial pressure of oxygen (P_{02}) in an oxygraph pig was measured using an YSI Model 5300 Biological Oxygen Monitor equipped with a platinum Clark electrode. 3.0 x 10^6 BT-474 cells were seeded in 10 mL of cell culture medium at 37°C prior to treatment. The cells were treated with DHA or an equal volume of EtOH 48 hours prior to the experiment. Non-treated cells were used as a control. The cells were trypsinized, counted, and resuspended in 37°C phosphate buffered saline (PBS) to a final concentration of 3.0 x 10^4 cells/ul. 100 ul of cell suspension was injected into a pig

containing 180 ul PBS equilibrated to 37°C. The rate of change in P_{02} was determined following successive treatments of glycolytic or mitochondrial inhibitors and electron donors. A complete profile of stepwise controlled ETC – complex linked metabolism would be generated by adding compounds in the following order: 2-deoxyglucose (2-DG), oligomycin A, carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone (FCCP), glutamate and malate, rotenone, succinate, malonate, glycerol-3 phosphate (G3P), antimycin A, N,N,N',N'-tetramethyl-1,4-benzenediamine dihydrochloride (TMPD) with ascorbate, and cyanide.

Inhibitor Preparations

The following stock solutions were prepared in water: 1 mM Rotenone; 1 mM FCCP; 10 mM Malonate; 10 mM Potassium Cyanide (KCN). Stock solution of 1 mM Oligomycin A and 1.0 mM Antimycin A were prepared in 95% EtOH. All inhibitor solutions were stored at -20 °C.

Substrate Preparations

The following stock solutions were prepared in water: 15 mM glutamate; 2.5 mM malate; 10 mM succinate; 20 mM Glycerol-3-Phospahte (G3P); and 50 uM ADP. Solutions of 10 mM ascorbate and 0.2 mM tetramethyl-p-phenylenediamine (TMPD) were made in 95% EtOH. All inhibitor solutions were stored at -20 °C.

Cell Viability Assay

Cells were trypsinized and counted using trypan blue staining and a hemacytometer.

Unstained cells were counted as viable

Data Recording and Analysis

All Oxygraph data was recorded and analyzed using WINDAQ32 analytic software

Results

The oxygraph measures real time oxygen concentration in the experimental vessel called a "pig." When cells are added to the pig, cellular oxygen consumption rate (OCR) can be estimated by monitoring real time change in oxygen concentration. OCR can be correlated to mitochondrial aerobic respiration. Every cell line has a unique metabolic profile that depends on a number of factors. One such factor is cell confluencey during tissue culture. In order to conduct constant and comparable oxygraph experiments an optimization control on cell confluencey must be performed.

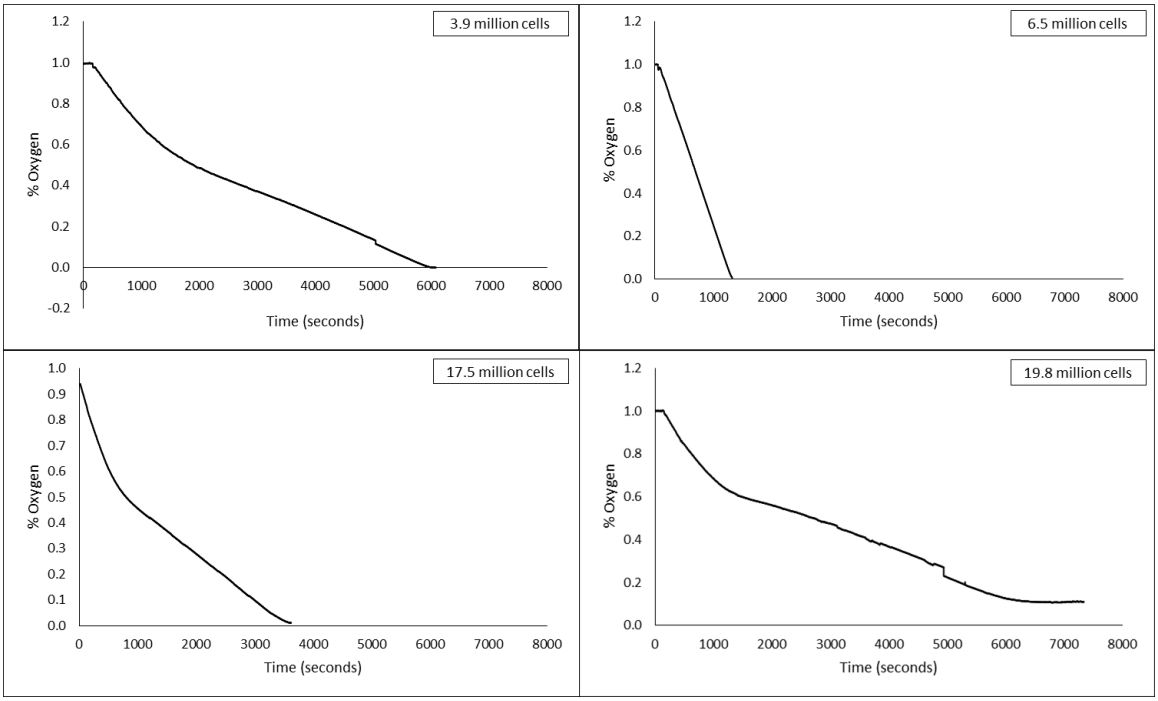

Figure 1: Cell confluency optimization for oxygraph. BT-474 cells were seeded in T75 cm^2 flasks at various densities. After 72 hours,3 million cells were resuspended in PBS and injected into the oxygraph.

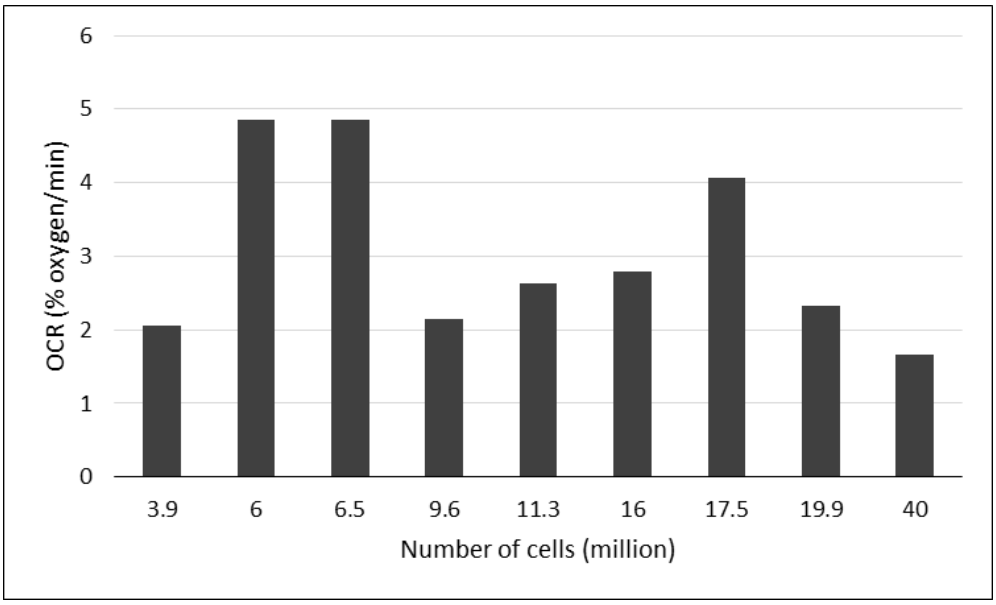

Figure 2: Oxygen consumption rate dependence on cell confluency. BT-474 cells were cultured at various densities and oxygen consumption rate was determined on the oxygraph. Maximal O_2 consumption rate was determined using WINDAQ32 data analytics software.

BT-474 cells were maintained with different cell densities and examined on the oxygraph for consistency (fig. 1) and maximal OCR (fig.2). 6-6.5 x 10^6 cells display the most consistent study system to study BT-474 cells. All cell counts above and below 6 million cells show a sharp change in OCR midway through the experiment which possibly indicates non-renewable substrate depletion (fig 1). This type of inconsistency is undesirable as it introduces unpredictable inconsistencies that would prevent accurate analysis of changes in OCR upon substrate addition in future experiments. Additionally, these cell concentrations exhibited decreased maximal OCR (fig 2) compared to the 6 million cell size sample. Results indicate that cells must be grown to a final confluencey of approximately 6 million cells per T75 cm^2 maintenance flask for optimal consistency and maximum OCR.

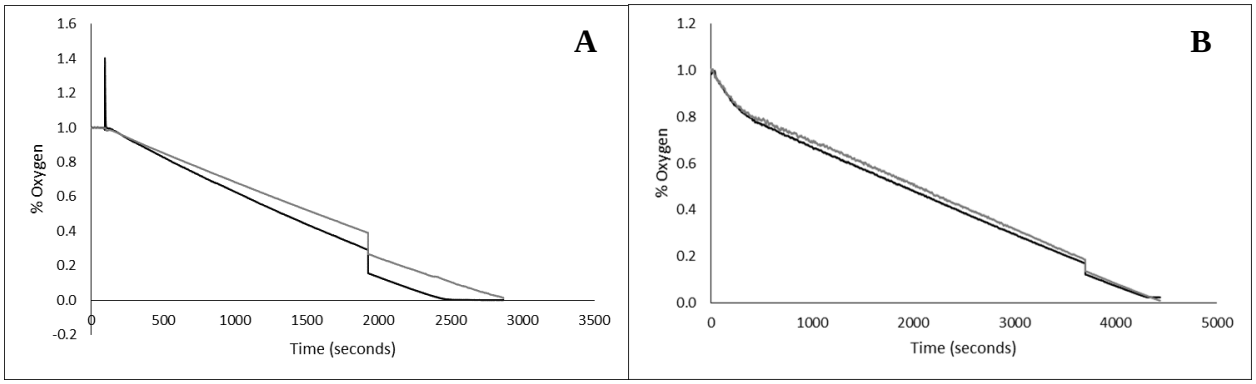

Figure 3: Two pig optimization. 3 million BT-474 cells from the same culture flask were added to two separate pigs and run simultaneously on the oxygraph. Each pig was stationed either on (A) different stir plates or (B) on the same stir plate. Oxygen consumption rates were recorded simultaneously.

A major limitation of using an oxygraph is the number of cells required for each experiment. Growing a sufficient number of cells can be both cumbersome and time consuming. Due to the fact that optimal cell confluency was determined to be 6 million cells (fig. 1) and 3 million cells are needed to for each experiment, it was realized that two experiments could be performed from a single flask of cells. In order to maximize efficiency, two pigs were set up to run in parallel. At first, the two pigs were placed on separate stir plates (fig. 3A). However, uncorrectable variations in stir bar speed resulted in non-comparable OCR readings between the two pigs. Placing both pigs on the same stir plate rectified this difference (fig. 3B). Effectively setting up two pigs to run in parallel doubles throughput for the oxygraph, reducing both cost and time expenditure, while maintaining data fidelity.

Table 1: Agents used to dissect ETC complex activities when using a Clark-type electrode

Agent	Description
Glutamate + Malate	Measures OCR from combined ETC complexes, I, III, and IV. Electrons from NADH generated from glutamate oxidation by glutamate dehydrogenase enter the respiratory chain at complex I.
Rotenone	Rotenone is an inhibitor of complex I. Addition of rotenone will recude OCR and show other oxygen consuming processes in the system.
Succinate	Succinate provides electrons directly to complex II and bypasses complex I of the ETC. OCR increase through complex II when cells are exposed to rotenone.
Malonate	Malonate competitively inhibits ETC complex II. Treatment with malonate indicates the extent of activity and intacktess of ETC complex II. Malonate is a reversible inhibitor.
Glycerol-3-Phosphate	G3P provides electrons for ETC complex III through the glycerol-phosphate cycle via glycerol-3-phosphate dehydrogenase. G3P bypasses blocked complexes I and II and increase OCR even after mitochondria are exposed to rotenone and malonate.
Antimycin A	Antimycin A inhibits complex III of the ETC. Addition of antimycin A after G3P is useful for determining OCR activity of ECT complex III.
Ascorbate + TMPD	TMPD is an artificial electron donor to ETC complex IV and ascorbate maintains TMPD in a reduced state. OCR increases through complex IV when mitochondria are also exposed to Antimycin A.
KCN	KCN irreversibly inhibits complex IV. Treatment with KCN will decrease OCR down to the instruments baseline.
ADP	ADP addition increases OCR in proportion to ADP addition when the ETC is coupled to ATP synthesis.
FCCP	ETC uncouples that dissapates mitochondrial inner membrane potential by acting as a protein ionophore that triggers nonspecific proton leak. FCCP is used to assess maximal respiratory capacity.
Oligomycin A	Oligomycin A is an antibiotic that blocks complex V (ATP synthase) of the ETC.

*Table was adapted from (Hong et al. 2012)

Specific mitochondrial inhibitors and substrates can be added to cells in order to stop or stimulate electron flow through each complex of the electron transport chain (Table 1). When added to cells in an oxygraph, these compounds can be used to determine the activity and intactness of each individual complex of the ETC.

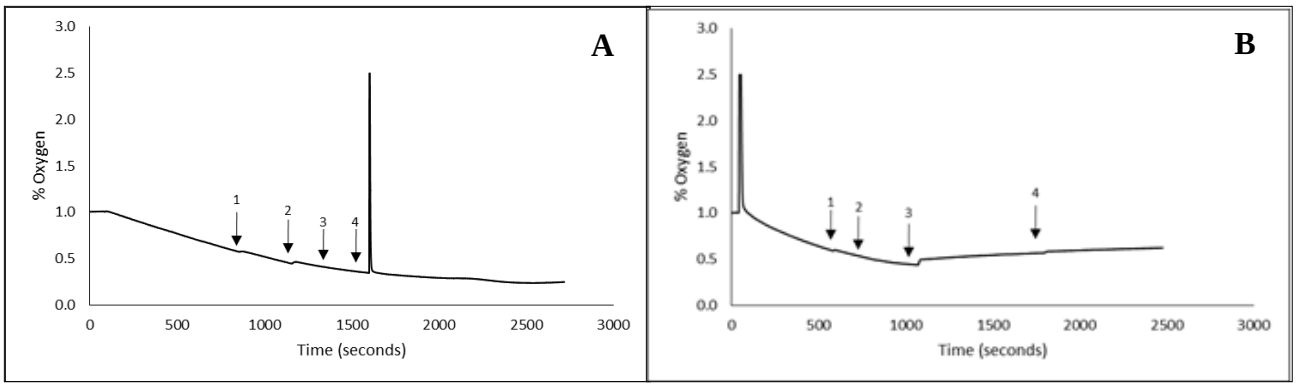

Figure 4: Mitochondrial substrates test. 3 million BT-474 cells were run in the oxygraph. The following substrates were added to A: (1) 20 uL 2-DG; (2) 100 uL Malate + Glutamate; (3) 100 uL Oligomycin A; (4) 100 uL FCCP and B: (1) 40 uL ADP; (2) 100 uL Glutamate + Malate; (3) 100 uL Rotenone; (4) 100 uL Succinate. Samples were run simultaneously.

To test the activity of mitochondrial targeted compounds, various substrates and inhibitors were added to untreated BT-474 cells (Fig 4A and 4B). Interestingly, none of the compounds worked as expected. Additions of 2-DG, Malate, Glutamate, FCCP, and ADP should have all stimulated OCR and caused the slope of the graph to decrease sharply. However, this was not observed. Conversely, Oligomycin A and Rotenone should have stopped OCR completely. Oligiomycin A did not inhibit OCR (Fig 4A) and rotenone seemed to actually increase the amount of oxygen present in the pig. Together these results suggest that the mitochondrial compounds are not working properly. Possible reasons for improper activity include membrane corruption or poisoning by substrate, OCR changes induced by ethanol used to dissolve some compounds, expired or defective compounds, or inability of the compound to reach the mitochondria.

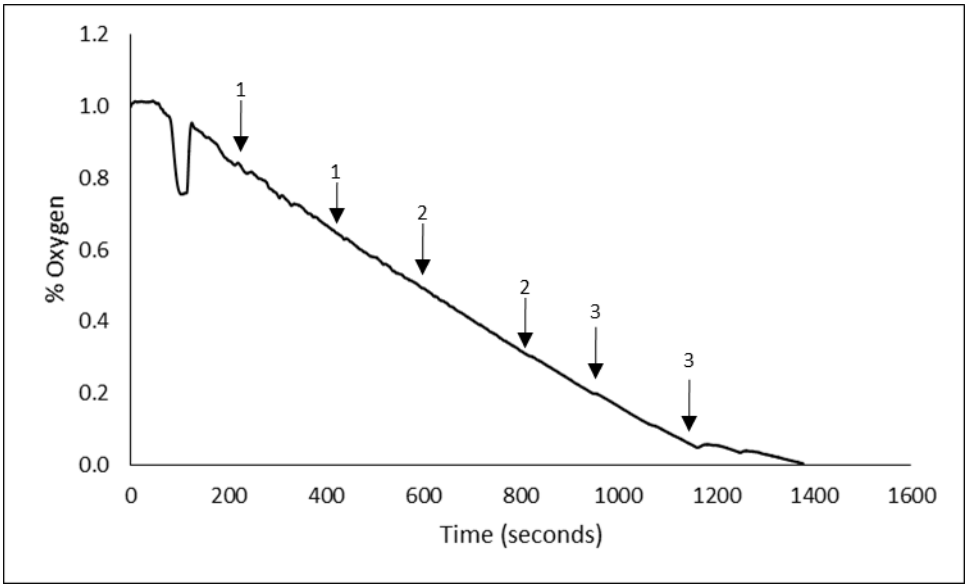

Figure 5: Ethanol's effect on respiration. 3 million BT-474 cells were run in the oxygraph. 95% Ethanol was added consecutively in various concentrations: (1) 10 uL EtOH; (2) 25 uL EtOH; (3) 50 uL EtOH. Experiment represents an N=3.

Some mitochondrial compounds were dissolved in ethanol. To determine if the ethanol was negatively affecting OCR, experiments were performed in which various amounts of 95% ethanol were added to untreated BT-474 cells. Results indicate EtOH does not affect OCR even at amounts up to 250 uL.

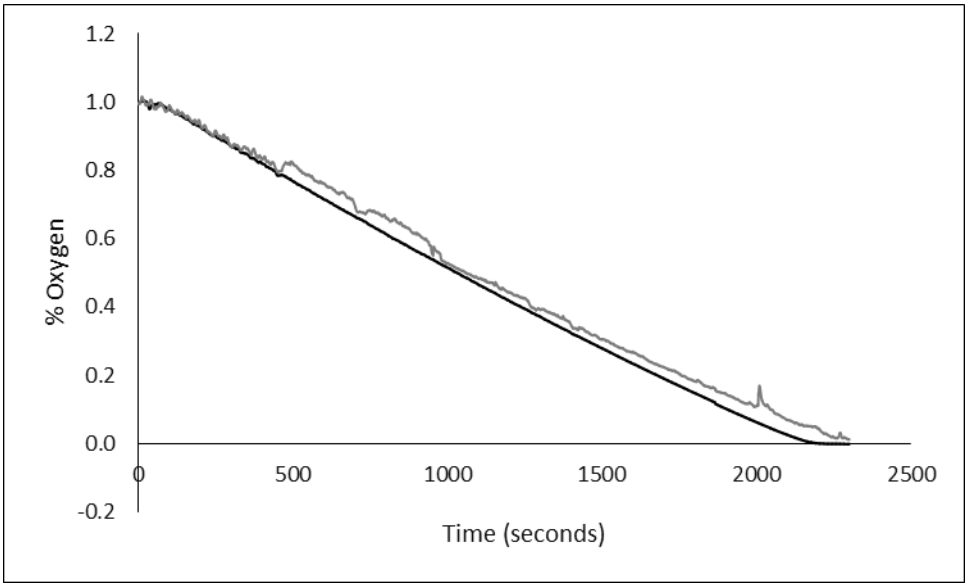

Figure 6: Digitonin's effect on respiration. 3 million BT-474 cells were run in parallel on the oxygraph. Solution 1 (grey) contained PBS. Solution 2 (black) contained PBS + 0.003 wt% digitonin.

Further research into the literature indicated that some mitochondrial substrates such as succinate and glutamate are unable to cross the plasma membrane of cells and therefore unable to reach the mitochondria, thus preventing them from properly affecting OCR. In an effort to circumvent this limitation, 0.003% digitionin was used to selectively permeabilize the plasma membrane. Digitonin is a molecular detergent that should allow all mitochondrial compounds to permeate the cellular membrane without issue. Before inhibitors could be tested, the effect of digitonin on basal OCR needed to be determined (fig. 6). Results indicate that addition of digitonin to BT-474 cells does not affect basal cellular respiration rates.

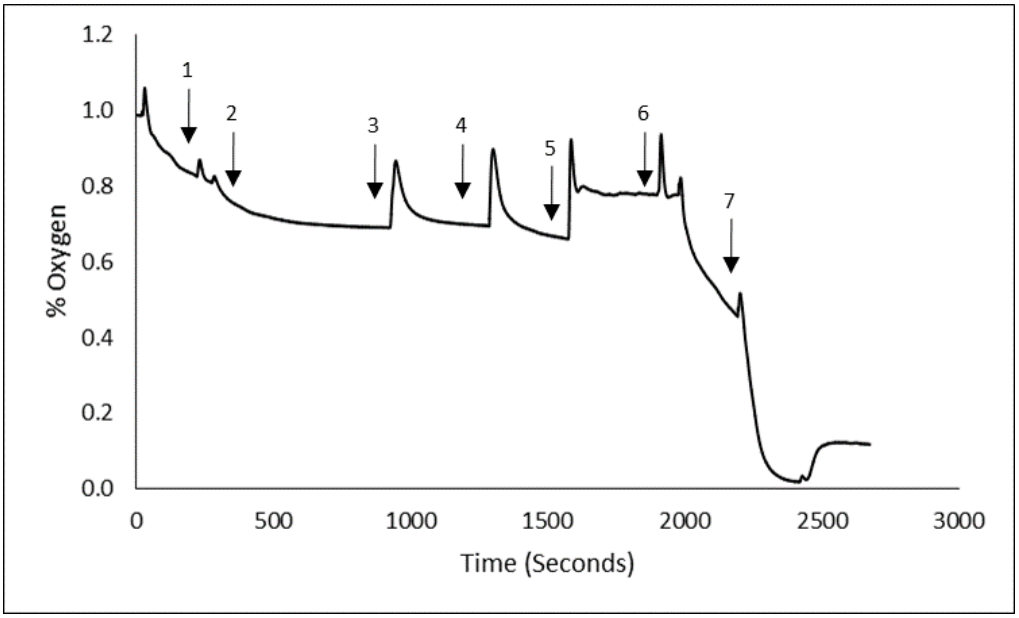

Figure 7: Mitochondrial compounds with diginonin permeabilized cells. 3 million BT-474 cells were run on the oxygraph. Substrates were added to assess metabolic activity: (1) 50 uL Glutamate + Malate; (2) 25 uL Rotenone; (3) 50 uL Succinate; (4) 100 uL succinate; (5) Stir bar activation for 2 seconds; (6) 100 uL TMPD + Ascorbate; (7) 100 uL sodium cyanide. Experiment was run simultaneously in duplicate.

To assess whether digitonin successfully permeabilized the plasma membrane so that the mitochondrial compounds could enter the cell, a gambit of substrates were tested with cells treated with 0.003% digitonin (fig. 7). Results indicate that the permiabilization of the cell membrane with digitonin was not sufficient to alter results upon the addition of most substrates. It is unclear if this is due to a possible sustained inability of substrates to penetrate the membrane. However, the slight increase of OCR upon succinate addition, and the drastic increase thereof with TMPD + ascorbate addition indicate that the substrates are passing through the membrane and reaching the ETC in mitochondria

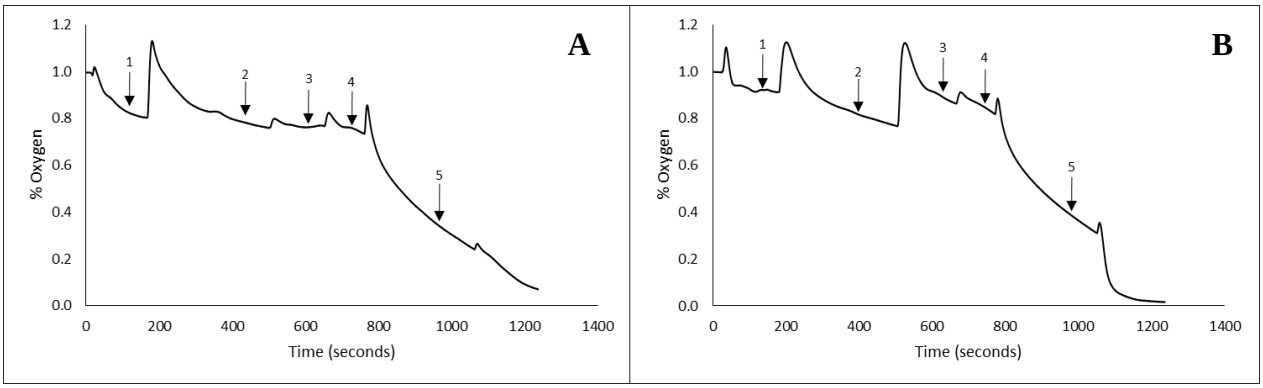

Figure 8: DHA's effect on complex IV activity. 4 million BT-474 cells were cultured with 165 uM DHA 48 hours prior to being run on the oxygraph. Substrates were added to determine metabolic activity: (1) 100 uL Rotenone; (2) 100 uL antimycin A; (3) 100 uL TMPD; (4) 100 uL Ascorbate; (5) 100 uL Sodium Cyanide. OCR was monitored simultaneously in DHA treated (A) and untreated (B) cells.

Activity of Complex IV of the electron transport chain activity in DHA treated and non-treated cells was assessed using the oxygraph. Rotenone and Antimycin A were added to block complex I and III respectively. TMPD and ascorbate are able to bypass complexes I, IIand III and feed electrons directly to complex IV. Adding rotenone and antiymicn A before TMPD and ascorbate ensures that all observed oxygen consumption is a result of activity directly though complex IV. When comparing treated to non-treated cell lines, the effect of TMPD and ascorbate were virtually identical. Following treatment with complex IV substrate, the DHA treated cells maintained an OCR of 2.78 % oxygen/min/3.0 $x10^6$ cells with the non-treated cells respired at 2.81 % oxygen/min/3.0 $x10^6$ cells. The identical values of these slopes indicate that that activity of complex IV is conserved between treated and non-treated cells which suggests that the decreased activity of oxidative phosphorylation seen upon DHA treatment is upstream of complex IV.

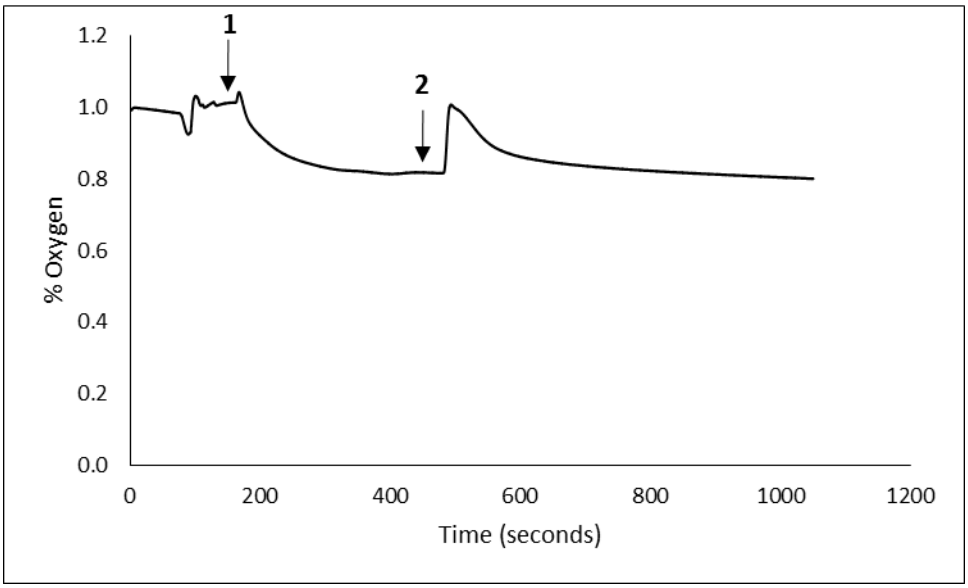

Figure 9: TMPD and Ascorbate Positive Control. OCR was monitored upon addition of (1) 100 uL TMPD and (2) 100 uL Ascorbate in the absence of cells.

In order to eliminate all possible confounds, a negative control in which TMPD and ascorbate were added to an oxygraph pig in the absence of cells. Surprisingly, addition of both TMPD and ascorbate caused the appearance of oxygen consumption. This result should not have been possible without cells present. While the OCR did not continue ad infinatum, this lends a generous amount of doubt into the results seen earlier in figure 8. It is impossible to know if the increased oxygen consumption was due only to complex IV activity or whether the TMPD and ascorbate mimicked OCR by some other means. Essentially, this control rendered all previous experiments performed with TMPD and ascorbate untrustworthy.

Discussion

The Oxygraph

The Oxygraph is a Clark-electrode type that can accurately determine the activity and intactness of each protein complex in the electron transport chain (ETC) of the mitochondria. It is advantageous over other metabolic profile measuring techniques in that it is flexible in measuring mitochondrial function, can accommodate a large number of substrate and inhibitor additions, and is relatively inexspensive. However, it is difficult to get the oxygraph to work properly in a consistent manner. A large amount of optimization and calibration must be performed in order to ensure it is working properly (fig. 1, 3-10). Additionally, sometimes oxygen leaks from the apparatus causing the oxygen consumption profile to read inaccurately and some drugs are not easily removed from the chamber and residues could potentially affect OCR. Furthermore, some drugs or solutions may even directly interact with the silver electrode to give the illusion that oxygen concentration is changing. For example, TMPD and ascorbate were shown to induce the appearance of oxygen consumption even in the absence of cells (fig. 9). While the ultimate change in oxygen concentration was not extreme and did level out over time, this inconsistency introduces a level of uncertainty into the results obtained when analyzing functionality of complex IV (fig. 8). TMPD and ascorbate bypass the rest of the ETC and donate electrons directly to complex IV. It appears that addition of TMPD and ascorbate stimulates OCR at the same rate in both treated and non-treated cells. This indicates that DHA treatment does not affect electron flow through complex IV and that the activity of complex IV is preserved (fig 8.) However, these results cannot be trusted as the change in OCR cannot be definitively proven to be from ETC

stimulation instead of background effects seen in fig 9.

Another limitation of the oxygraph is the limit of detection, as it has a much more narrow range and higher oxygen consumption rate than some newer technologies. Furthermore, the oxygraph requires cells be in suspension, which is postulated to change signaling and potentially bioenergetics in comparison to new technologies which measure OCR directly from the culture vessel. Originally, this study was designed to compare the two metabolically distinct cancer cell types BT-474 and MDA-MB-231 with a non-transformed cell line MCF-10A. BT-474s are mitochondrially active, MDA-MB-231 cells are glycolytically active, and MCF-10As represent a normal phenotype. Unfortunately, only the BT-474 cells had sufficiently robust respiration levels to be registered on the oxygraph. Both the MCF-10A and MDA-MB-231 cells did not show any oxygen consumption. The inability to compare results across various cell lines severely hampers the integrity of the study.

Ultimately, the oxygraph represents a promising and exciting method for measuring ETC complex activity. However, too much optimization of technique is required in order to obtain consistent and trustable results. So far it has not been feasible for the BT-474 cells. All thing are considered, other techniques like the XF24 extracellular flux analyzer might be a better option to continue this research. Although each run is significantly more expensive on the XF24 analyzer, it has a well-established protocol that runs consistently and could save both time and money by reducing the amount of optimization that needs to be done.

Mitochondria and Cancer

Otto Warburg was the first person to recognize that cancer cells prefer using aerobic glycolysis over oxidative phosphorylation to generate ATP. Recent studies indicate that carcinogenesis might be a result of this deregulated cellular metabolism [11]. Numerous studies show that cancer cells have a structurally unstable or non-functional mitochondrial that are unable to generate sufficient levels of ATP to maintain cellular needs [12-16]. New focus has been applied on compounds that target cancer metabolism as a method of treatment.

Dietary lipids have been extensively studied in association with breast cancer survival and recurrence [17]. Specifically, the balance and source of dietary lipids have been shown to play an important role in cancer risk [18]. Omega-3 Polyunsaturated fatty acids (PUFA) have been shown to positively reduce malignant transformation, angiogenesis, and tumor cell growth in cell culture. Of the omega-3 PUFAs, docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA) are thought to exhibit the largest anti-cancer effects. Recent studies have shown that treatment with DHA decreases ATP production through both glycolysis and oxidative phosphorylation in mammary cancer cells [11]. Importantly, maximal oxygen consumption rate has been shown to be decreased upon DHA treatment, indicating that DHA significantly alters the activity of the ETC whether directly or through a metabolite.

There are various mechanisms through with DHA could be exhibiting its effects. DHA could be altering global ETC function by altering the fatty acid composition of the mitochondrial membrane. DHA is a long chain PUFA with six double bonds. It is thought to adopt a kinked 3-dimensional structure which causes an increase in membrane fluidity upon DHA treatment. In the plasma membrane, incorporation of DHA has been

shown to disrupt lipid rafts and alter cellular signaling [20] It is postulated that mitochondria might contain lipid raft like domains that help organize proteins in the inner mitochondrial membrane. It is possible that DHA could be interfering with these mitochondrial domains in a similar fashion thus altering metabolic function [20]. Evidence indicates that in the mitochondria, dietary fish oil induces uncoupled respiration (proton leak) in colon cancer cells [21]. This may be a result of DHA preferentially incorporating into mitochondrial cardiolipin [22], [23]. Because the ETC relies upon a proton gradient to generate ATP, it is possible that DHA incorporation into mitochondrial membrane induces proton leak in the mitochondrial membrane and therefore decreases maximal respiratory capacity. This theory might also explain why DHA treatment results in increased in reactive oxygen species (ROS) because most cellular reactive oxygen species are generated as electrons leak out of the ETC and interact directly with oxygen.

It is also possible that DHA affects the ETC by acting through electron transferring flavoproteins (ETF). ETFs are soluble mitochondrial matrix proteins that utilize energy generated from β-oxidation of fatty acids (like DHA) to transfer electrons directly to complex III of the ETC. It is possible that DHA treatment alters the functionality of ETFs thereby decreasing overall oxygen consumption capacity although the activity of EFTs have never been studied in relation to DHA supplementation.

As discussed above, results indicate that the activity and intactness of complex IV is unaffected by DHA treatment (fig. 8). If DHA were significantly altering mitochondrial membrane fluidity and causing electron leak, the OCR from DHA treated cells would be significantly slower than non-treated cells. However, this was not the case, implying that DHA works via a focused effort on a specific ETC complex or

combination of complexes instead of affecting the entire ETC by altering membrane functionality. Unfortunately, due to the questionable nature of the control (fig. 10) it is impossible to declare for certain whether complex IV really wasn't affected by DHA treatment.

As a benign dietary substance, DHA treatment may be used as an effective non-invasive therapy. Previous data show that DHA coordinates integrated targeting of major signal-transduction pathways and metabolism in parallel [9]. DHA is not powerful enough to treat cancer on its own. However, it can be used as a useful supplement to enhance other treatments. DHA has been shown to enhance efficacy in combination with chemotherapies [24]. For example, unpublished data from our lab indicates that combination treatments with 2-DG and DHA enhanced metabolic injury to the cancer cells (unpublished data). A better understanding of the mechanism by which DHA acts will help us be able to better identify metabolically targeted compounds that could synergize with DHA providing innovative new strategies in combination therapies to target cell metabolism.

Future Directions

The next step of this project will be to finish optimizing the oxygraph. Or, if that proves impossible, move on to another technique such as single flow mitochondrial analysis or XF24 extracellular flux analysis to determine the locus at which DHA acts on the ETC. Once DHA's mechanism of action is identified, studies will be performed with various metabolic inhibitors in hopes of finding a synergistic combination.

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