An Attempt to Reverse Aspects of the Warburg Effect Using 17 β-estradiol

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Dedication

To my family and friends.

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Abstract

The Warburg effect is defined as the propensity for cancer cells to favor glycolysis over oxidative phosphorylation under aerobic conditions. Finding a way to reverse this effect would likely be very beneficial for cancer therapy. The PI3K/Akt pathway has been suggested to be responsible for the Warburg effect, and estrogen is a known regulator of this pathway. Estrogen, specifically 17 β-estradiol, has been shown to be protective at the level of the mitochondria. The purpose of this study was to try to use 17 β-estradiol to reverse aspects of the Warburg effect in two cancer lines. Various concentrations of 17 β-estradiol were added to the samples (0, 10nm, 100nm, 1μm) for various amounts of time (16-96h). Western blots probes for select subunits of the electron transport chain (ETC) showed no differences in cells with and without 17 β-estradiol across various times. Due to technical difficulties with cell lines, considerable troubleshooting was required, consuming the time available for further analysis. The available results do not suggest that 17 β-estradiol alone is able to reverse the Warburg effect.

Table of Contents

I. Title…………………………………………………page 1

II. Dedication…………………………………………page 2

III. Acknowledgements………………………………page 3

IV. Abstract……………………………………….…….page 4

V. Table of Contents…………………………….……page 5

VI. Images…………………………………………….…pages 12 & 21

VII. Introduction……………………………….….……pages 6-16

VIII. Methods……………………………………….…….pages 17-19

IX. Results………………………………………….……page 20-21

X. Discussion……………………………………….……pages 22-26

XI. Conclusions/Future aims………….…………..……page 27

XII. References…………………………………..………pages 28-39

Introduction:

The "Warburg effect," initially described by Dr. Otto Warburg in the 1920s, states that rapidly dividing tumor cells consume glucose at a much higher rate than normal cells and use the glucose to produce lactate rather than perform oxidative phosphorylation even when plentiful oxygen is available (Warburg 1927, Warburg 1956). This effect has also been referred to as "aerobic glycolysis." In noncancerous cells, the availability of oxygen typically enhances oxidative phosphorylation.

Cells normally have two major ways to obtain energy from glucose: aerobic respiration and fermentation to lactic acid. Aerobic respiration is the more energy-efficient use of glucose but only occurs when adequate oxygen is available. In this process, glucose is oxidized to carbon dioxide and water. Glucose derivatives feed the tricarboxylic acid (TCA) cycle as well as oxidative phosphorylation, and oxygen is able to form 36 ATP per glucose molecule. Fermentation to lactate is able to produce a large amount of ATP as well, but only at the expense of larger amounts of glucose. This process converts glucose to pyruvate, which is reduced to lactate, making only 2 net ATP per glucose, making it much less energy efficient.

Warburg first discovered aerobic glycolysis using rat liver carcinoma (Warburg 1924). He found that while these cells did not consume more oxygen than normal, noncancerous cells, they did produce more lactate when oxygen was available (Warburg 1925, Warburg 1956). This is unlike normal cells, which produce almost no lactate in the presence of oxygen. In the 1950s, Warburg used mouse ascites tumor cells and observed that these cells produced more lactate than the previous cancer cells (Warburg 1956). This observation was expanded to human carcinomas (Cori & Cori 1925). In vivo studies have shown 2/3 of glucose consumed by tumor cells was converted to lactic acid under aerobic conditions, where healthy cells produced no lactic acid under the same conditions; also the consumption of glucose in tumor cells was elevated (Warburg 1926, Warburg 1927). The increase in glucose utilization, up to tenfold higher than normal cells (Warburg & Minami 1923), described by Warburg is the basis for the modern use of PET scans in detecting cancers (Pauwels *et al.* 2000). Studies using chickens with tumors in only one wing showed increase in lactate and decrease in glucose and carbon dioxide in the tumorous veins compared to the control veins (Cori & Cori 1925), adding further proof to the theory.

The observation by Warburg that cancerous cells perform such inefficient energy metabolism has puzzled scientists for decades. Why would cells that require a large amount of energy, use such a wasteful form of metabolism? This seems to be a disadvantage; rapidly proliferating cells, those found during embryogenesis, growth, and tumorigenesis, require high amounts of ATP for proliferation (DeBeradinis *et al.* 2007). Warburg initially proposed that there was a defect within the mitochondria of tumor cells and they were *unable* to use oxygen to produce ATP (Warburg 1956). He thought this might be the cause of the tumor rather than the effect. This belief has been largely disproven, as cancer cells are able to use oxidative phosphorylation in addition to the favored aerobic glycolysis and are without defects in oxidative metabolism (Moreno-Sanchez *et al.* 2007). Further studies suggested that perhaps this mechanism allows cancer cells to continue to grow when placed in a hypoxic environment (Weljie & Jirik 2011), and perhaps this defense mechanism allows more aggressive forms of the tumor to thrive (Gatenby & Gillies 2004). However, this does not explain why these cells still undergo aerobic glycolysis when adequate oxygen is available. A more likely theory is that cancerous cells may favor aerobic glycolysis because of the large

number of carbon-based intermediates that are made (Vander Heiden *et al.* 2009), which can be used to support proliferation. The process of cell division requires more than just ATP. It requires each cell to double total biomass in order to divide and produce two equal daughter cells. Intermediates formed during glycolysis from glucose can be used to make lipids, proteins, and nucleic acids, all of which can be used for cellular proliferation. While oxidative phosphorylation is able to make the most energy from glucose (36 ATP per glucose), aerobic glycolysis can actually produce more energy in the same amount of time (56 ATP from 11 glucose) making 10% more ATP than normal cells (Warburg 1926), even though each glucose is only able to produce 2 ATP (Guppy & Greiner 1933). This may seem wasteful but each lactate produced leaves three carbons that can be used for incorporation into new cell biomass (Vander Heiden *et al.* 2009). If nutrients are readily available, as is usually the case in multicellular organisms, then cells do not necessarily need to ration their supplies and perform oxidative phosphorylation—glucose is plentiful. Oxidative phosphorylation also has disadvantages. Although energetically favorable, production of reactive oxygen species (ROS) in the ETC can lead to cellular damage (Valko *et al.* 2006).

Studies suggest that Akt, a serine/threonine kinase, may be responsible for initiating and maintaining the Warburg effect (Robey & Hay 2009). Akt, also known as protein kinase B (PKB), is one of the most activated oncoproteins in human cancer cells (Osaki & Oshimura 2004, Bhaskar & Hay 2007). Akt, a downstream effector of the phosphatidylinositol 3-kinase (PI3K) signaling pathway (Franke & Yang 1995) has many downstream effects related to cell survival and growth (see Image 1;Dudek *et al.* 1997, Bhaskar & Hay 2007). Activation of this pathway enhances many of the metabolic activities that support rapid proliferation. Through the PI3K pathway, Akt inhibits apoptosis (Downward 2004, Yao & Cooper 1995). Activation of this pathway leads to increased uptake of amino acids and increases lipid synthesis needed for proliferation, and directs the amino acids into protein synthesis (Bauer *et al.* 2005, Chang *et al.* 2005). Mammalian cells with increased levels of Akt have increased levels of ATP by as much as two- to threefold (Gottlob *et al.* 2001) and those with decreased Akt have lower levels of ATP (Gottlob *et al.* 2001). Most importantly for this paper, the increases in ATP caused by Akt are caused by both increased glycolysis (Elstrom *et al.* 2004) and oxidative phosphorylation (Robey & Hay 2009). The mechanisms

through which Akt does this are still largely unknown. This same pathway also helps to regulate the uptake and use of glucose through both glucose transporter-dependent and -independent pathways (Robey & Hay 2009). Akt does this by increasing the expression of glucose transporters and promoting their translocation to the plasma membrane (Wang *et al.* 1999).

Image 1:

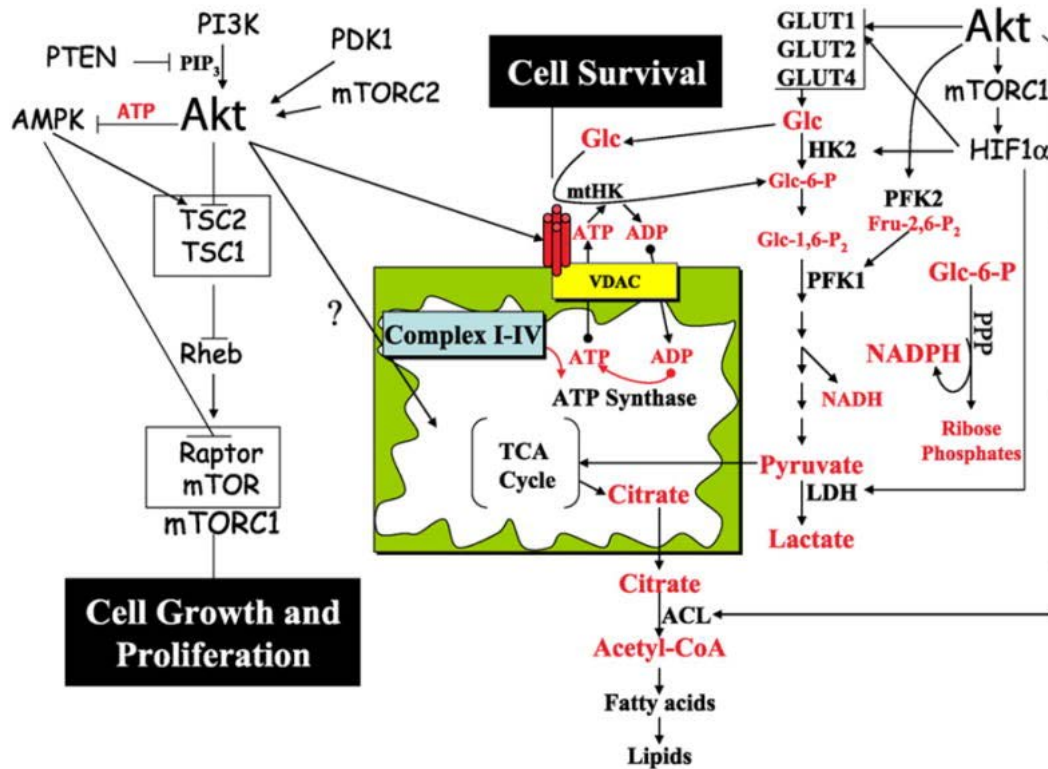


*Image taken from Robey & Hay in "Is Akt the Warburg kinase"?

Once Akt is activated, it increases ATP production by increasing oxidative phosphorylation and glycolysis. Akt increases cell survival. Akt also increases glucose uptake by increasing the expression of Gluc transporters. PI3K activation of Akt is responsible for the contributions to cell growth, cell proliferation, and susceptibility to oncogenic transformation.

The PI3K/Akt pathway can be activated in many different ways, among them, via 17 β-estradiol (Kazi *et al.* 2009, Zhao *et al.* 2008). 17 β-estradiol has been shown to be protective in healthy cells (Brinton 2005), and this protective effect of 17 β-estradiol has been extensively studied in relation to Alzheimer's disease (AD). Aβ, a pathologic hallmark of the disease, leads to an increase in ROS, increasing cell stress, and resulting in cell apoptosis (Nilsen *et al.* 2006). However, pre-treatment with 17 β-estradiol prior to Aβ insult, leads to protection in vivo and in vitro (Brinton *et al.* 1997, Nilsen *et al.* 2006). Many of the protective mechanisms attributed to 17 β-estradiol have been shown to converge on the mitochondria (Nilsen & Brinton 2004). 17 β-estradiol protects cells against apoptosis (Nilsen *et al.* 2006). In AD, there is a downregulation of the anti-apoptotic protein, Bcl-2, and mitochondrial translocation of a protein known to promote cell death, Bax, leading to release of cytochrome c, and subsequent cell death. Pretreatment of cells with 17 β-estradiol prior to Aβ insult prevents all of these processes leading to decreases in apoptosis (Nilsen *et al.* 2006). 17 β-estradiol pretreatment leads to decreases in ROS generation (Nilsen *et al.* 2006). Aβ also increases intracellular calcium (LaFerla 2002). Calcium is important in many cell signaling cascades and tight

regulation is required for healthy cells. 17 β-estradiol protects against the rise in intracellular calcium and maintains calcium homeostasis, further suggesting that 17 β-estradiol protection works at the level of the mitochondria, which buffer excess calcium (Nilsen 2004, Nilsen *et al.* 2006). 17 β-estradiol pretreatment has been shown to increase ATP production in healthy neurons and sustain ATP production after insults (Brinton *et al.* 2000). Female rats treated with 17 β-estradiol also have increases in mitochondrial proteins involved in cellular energy, free radical maintenance, metabolism, and cell survival (Nilsen *et al.* 2007), leading to improved utilization of glucose through oxidative phosphorylation. Specifically they show increases in proteins involved in complexes IV and V of the ETC (Nilsen *et al.* 2007).

This protection by 17 β-estradiol occurring at the level of the mitochondria happens through activation of the PI3K/Akt pathway (Mannella & Brinton 2006). 17 β-estradiol activates this pathway, which also results in cell proliferation (Zhao *et al.* 2008). 17 β-estradiol also upregulates hexokinase, which may be responsible for some of the anti-apoptotic properties of Akt (Gottlob 2001). 17 β-estradiol also increases levels of insulin like growth factor-1 (IGF-1), which also further activates the PI3K/Akt pathway (Simpkim 2008).

Cancer cells have multiple mutations leading to inactivation of the tumor suppressor PTEN, resulting in uncontrolled Akt activation (Robey & Hay 2009). This activation of Akt drives glycolysis and lactate production and increases glucose uptake (Fan 2010). In noncancerous cells, continuous activation of Akt leads to feedback inhibition of the pathway at the level of PI3K (Shineman *et al.* 2009). However this inhibition appears to be lost in most cancer cell lines. Some molecules have been shown to disrupt the PI3K signaling pathway and lead to decreased glucose uptake by tumors (Vander Heiden *et al.* 2009). By targeting this pathway we may be able to disrupt cancer (Robey & Hay 2009). The protective effects of 17 β-estradiol on mitochondrial function and its ability to regulate the PI3K/Akt pathway suggest that it might be able to disrupt this signaling pathway. Its impact on the mitochondria at the level of respiration suggests that 17 β-estradiol use may be protective against tumor cells using aerobic glycolysis and 17 β-estradiol could possibly lead to increases in oxidative phosphorylation.

The goal of the current study was to define and try to reverse aspects of the Warburg effect in two standard cancer cell lines using 17 β-estradiol. We showed that both of our cancer cell lines downregulate

enzyme complex subunits in the ETC, without any compensatory increases in other complexes that would suggest mitochondrial dysfunction, consistent with the Warburg effect.

Methods

Cell culture:

Human Ntera-2 (NT-2) carcinoma and SH-SY5Y cell lines (American Type Culture Collection) were used in these experiments. The NT-2 line was isolated from testicular carcinoma, and the SH-SY5Y line was derived from neuroblastoma metastases. Both lines express characteristics of neuronal precursor cells, and can be differentiated into a fuller neuronal phenotype via application of retinoic acid. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) providing nutrients. Penicillin (5 U/ml) and streptomycin (5mg/ml) were used to prevent bacterial growth.

Cells were maintained in a flask and were incubated at 37°C in a 5% CO_2 atmosphere. Media was changed as needed. When flasks appeared 80-100% confluent cells were passed to new flasks at lower concentrations to prevent restriction of cell growth.

17 β-estradiol treatments:

Varying concentrations of 17-β-estradiol were used for this experiment. The concentrations of 17 β-estradiol added to the samples were 0

(vehicle [DMSO] control), 10 nm, 100 nm, and 1 μm. Previous studies suggest that concentrations greater than 10 nm have no added benefit or adverse effects (Brinton *et al.* 1997), but other studies have required doses up to 1 μm to see a response (Brinton 1991). Doses of 1 μm are considered high concentration. The smaller doses used are more physiologic (Brinton 1991). 17 β-estradiol treatment was done for various amounts of time (16-96 hours); 24 hours of 17 β-estradiol exposure has been shown to have a substantial impact on both growth and survival of neurons (Brinton *et al.* 1997). The same concentrations were added to all cell lines (Ntera-2, SH-SY5Y, RA-differentiated Ntera-2, and RA-differentiated SH-SY5Y cells).

Western Blots:

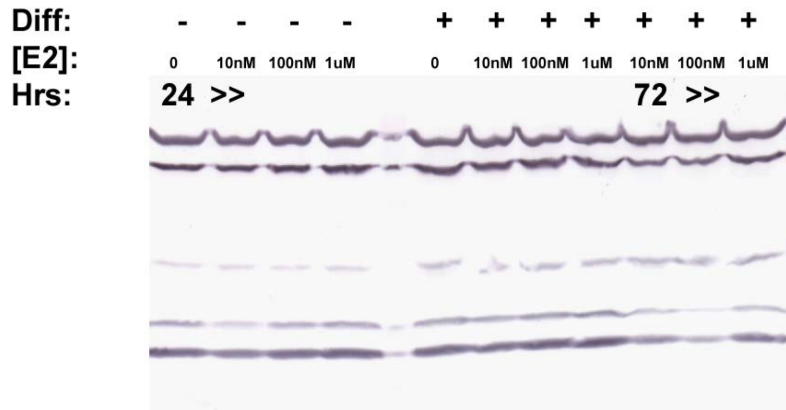
Cultured cells were suspended and washed with ice-cold PBS, pelleted, and resuspended with RIPA buffer (0.5% deoxycholate, 0.1% SDS, 1% NP40, 5mM EDTA in 50 mM Tris, pH 8.0) containing a protease inhibitor cocktail. Following five minutes of incubation on ice, the cellular debris was pelleted and supernatant stored at -80°C. Samples were loaded and run on a 10-20% 1.5mm SDS-PAGE Tris-glycine gel at 150 volts for 2 hours. Proteins were then transferred to a

PVDF membrane, fully submerged in CAPS transfer buffer (pH 11) with 10% methanol using 150 mAmps for 2 hours. Membranes were then blocked overnight in 5% Carnation dry milk in PBS+0.01% Tween-20 at 4°C. The ETC subunits assessed were complex I subunit NDUFB8 (approximately 20kD), complex II subunit 30kDa (approximately 30kD), complex III subunit core 2 (approximately 48kD), complex IV subunit II (approximately 24kD), and ATP synthase subunit alpha (approximately 55kD). (OXPHOS antibody cocktail, Mitosciences, Eugene OR at 1:1000 dilution), incubated for 2 hours. Secondary antibody was anti-mouse Alexa-Fluor 594 at 1:5000 dilution, incubated for 2 hours. The fluorescent bands were then visualized on a UVP blot imager.

Results

We previously showed that both the Ntera-2 cell line and SH-SY5Y cell line as well as their retinoic acid (RA)-differentiated lines downregulate many of the proteins subunits of the ETC, perform less oxidative phosphorylation, and yet maintain other aspects of mitochondrial function. Our goal was to stimulate increased oxidative phosphorylation by increasing expression of ETC proteins. However, we found no indication that the estrogen treatments had the desired effect. There were no apparent differences observed in any of the cells treated with estrogen for 16 h vs. 24 h vs. 72 h vs. 96 h. There were no differences observed between the cells treated without 17 β-estradiol (the vehicle control) and those treated with the different concentrations (10 nm, 100 nm, 1 μm). Image 2 shows a typical result from one Western blot. Due to technical difficulties with the cell lines and the initial blots, we did engage in considerable troubleshooting of our method, which ultimately consumed the time we had available for further analysis of the hypothesized pathway.

Image 2:



Western blotting results from one of the final experiments testing 17 β-estradiol-induced reversal of the Warburg effect. SH-SY5Y cells, proliferating as well as differentiated, were harvested after 24 or 72 hours of 17 β-estradiol treatment at various concentrations. Neither increased 17 β-estradiol concentration, from 10 nM to 1 μM, nor increased time of treatment, significantly increased the expression of the ETC subunits assessed in proliferating or RA-differentiated cells. Bands are as follows: Uppermost: ATP synthase subunit alpha, approx 55kD; Complex III subunit Core 2, approx 48kD; Complex II subunit 30kDa, approx 30kD; Complex IV subunit II, approx 24kD; Complex I subunit NDUFB8, approx 20kD.

Discussion

This study was designed to use 17 β-estradiol to try to reverse aspects of the Warburg effect, the observation that cancer cells undergo lactic fermentation rather than oxidative phosphorylation in the presence of adequate oxygen (Warburg 1927, Warburg 1956). This effect occurs without an obvious defect in the mitochondrial machinery. Both cancer cell lines, the Ntera-2 and SH-SY5Y, downregulated enzyme complex subunits in the ETC when compared to normal brain, resulting in decreased oxidative phosphorylation, but without indication of an intrinsic mitochondrial defect, consistent with the Warburg effect.

The Warburg effect, or aerobic glycolysis, is likely regulated by the kinase Akt (Robey & Hay 2009). Akt has been shown to be mutated in multiple cancer lines (Osaki & Oshimura 2004) and leads to both the increase in aerobic glycolysis (Elstrom *et al.* 2004) and the increase in glucose uptake observed by cancer cells (Robey & Hay 2009). In normal cells, continuous activation of Akt leads to a feedback inhibition of the PI3K pathway (Shineman *et al.* 2009). However, this is not observed in most cancer cells, which have mutations resulting in chronic activation of the PI3K/Akt pathway, with no feedback

inhibition. Estrogen, a known activator of this pathway (Zhao *et al.* 2008, Mannella & Brinton 2006), has been shown to be protective at the level of the mitochondria (Nilsen & Brinton 2004, Mannella & Brinton 2006, Nilsen *et al.* 2006). Our hypothesis was that estrogen would be able to alter regulation to again favor oxidative phosphorylation in the face of adequate oxygen, like normal cells under aerobic conditions.

Our results did not show any difference between cells treated with estrogen compared to those not treated (Image 2). They show no difference between the different concentrations of estrogen used (Image 2). These results were consistent between the two lines used for these experiments.

The results suggest that estrogen cannot reverse the Warburg effect, but there are multiple possibilities to explain the negative results observed in this experiment. Some can be explained by lab technique; we had to engage in extensive troubleshooting at the beginning of the experiment to establish protocols for sample preparation and blotting and may yet have unresolved technical issues that are confounding the results. Another explanation may be that the concentrations of estrogen were too low. However, these are the

concentrations frequently used in this type of study (Brinton 1991, Brinton *et al.* 1997).

Since multiple studies suggest that estrogen is protective/enhancing at the level of the mitochondria, including at the level of the ETC, we expected it may upregulate the expression of ETC subunits, but perhaps 17 β-estradiol works through a pathway not evaluated by this study. The goal of our study was only to examine ETC subunit expression after 17 β-estradiol treatment; it remains possible that 17 β-estradiol effects ETC function via other mechanisms not addressed here. Also, 17 β-estradiol has been shown to be beneficial largely only in healthy cells (Brinton *et al.* 1997, Nilsen *et al.* 2006), and has not been shown to be protective if used after an insult has occurred (Brinton 2005). The cells used in this experiment were cancerous cells, carrying significant chromosomal mutations. In this case it may be too late for estrogen to have a protective or compensative effect.

A mutation in the Akt pathway is observed in the majority of cancer cells (Robey &Hay 2009). In normal cells we know that over-activation of this pathway will actually feedback and inhibit the pathway (Shineman *et al.* 2009), however one of the hallmarks of

cancer cells is that they are able to evade normal feedback. Estrogen may be unable to work on this pathway once it has been mutated.

Another possibility for the negative results stems for the fact that there are three known isoforms of Akt (Bhaskar & Hay 2007). Akt1 in majority of tissues, Akt2 in insulin-responsive tissues, Akt3 in brain and testes. We know estrogen can activate Akt but perhaps it is unable to activate all of the isoforms and those used by tumors are different. Estrogen may selectively affect Akt isoforms that are not relevant in these cancer lines.

Decades of research, including that mentioned above shows that cancer cells favor aerobic glycolysis; however, some experts still disagree. Some studies suggest that malignant cells are not more glycolytic than normal cells (Zu & Guppy 2004); the Warburg effect may only occur as a result of certain common, but not unavoidable, mutations, and thus some cancers will not demonstrate the effect. Our previous research indicating that these cells perform consistent with the Warburg effect may be an overgeneralization.

Another possibility which was briefly mentioned above is that the Warburg effect has been studied in more than just cancer cells and it is possible that all rapidly dividing cells (embryogenesis,

tumorigenesis) may favor aerobic glycolysis (DeBerardinis *et al.* 2008) and that reversal is not possible in rapidly dividing cells that may require the carbon mass acquired via lactic fermentation for anabolic processes. Reversal of such a "normal" situation would not be favored under any conditions, particularly under presumably physiologic levels of endogenous hormone.

Conclusions/Future Aims

In conclusion, our results indicated a failure of 17 β-estradiol to enhance the expression of ETC protein subunits in our cancer cell lines, but a number of technical difficulties may have confounded our outcomes, and further investigation would be required to determine whether our hypothesis is correct or false. For instance, we planned to probe for Akt expression and activation in our treated cells, as well as probe for other confirmatory effects of estrogen treatment. Considerable work remains in order to determine whether this bioenergetic effect can be reversed, in vitro and ultimately in vivo, and whether doing so would have a beneficial effect on patient outcomes.

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