Pyruvate Dehydrogenase Phosphorylation in Tamoxifen Resistant Breast Cancer Cells

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Abstract
Pyruvate is converted to acetyl coenzyme A (Acetyl CoA) through the pyruvate dehydrogenase complex (PDC), a multi enzyme complex in cellular metabolism linking glycolysis and the citric acid cycle. The reversible phosphorylation of Pyruvate dehydrogenase (PDH) is an important step in the PDC complex in determining the fate of the metabolic process. Tamoxifen resistant (TAMR-MCF-7) human breast cancer cells have high activity of PDH and high mRNA expression of pyruvate dehydrogenase Kinase-4 (PDK4), an inhibitor of PDH (Walter et al. 2015). We hypothesize that the phosphorylation state of PDH is altered in tamoxifen resistant cells. To determine the phosphorylation state of PDH we used a phosphor-specific western blotting technique. Anti-phospho PDHE1-AType1(Ser293)) produced by Rardin et al that recognize the inhibitory phosphorylation sites in PDH was used, along with a PDH antibody that is not phosphorylation dependent, to observe if PDH is hypo or hyper phosphorylated.

Introduction
Breast cancer develops in women’s breast tissues. According to the American Cancer Society, it is the second leading cause of death in women in America, with an average of 40,000 deaths a year in the United States (Chang, 2012. Clarke et al, 2015). There are four different types of breast cancer; luminal A (estrogen receptor positive (ER+), luminal B, Her2+, and triple negative (ER-α, HER2+, and progesterone receptor negative) (Clarke et al, 2015, p. 1). Luminal A cancer is the most common and its proliferation is driven by estrogen. Tamoxifen, which is a selective estrogen receptor modulator (SERM), has been used for over 30 years in breast cancer treatment and breast cancer prevention in women at high risk for disease (Chang, 2012). Tamoxifen blocks estrogen signaling as well as blocks cancer cell growth, and it is effective in preventing cancer recurrence. However, twenty percent of patients who receive tamoxifen treatment develop tamoxifen resistant cancers (Walter et al, 2015).
When most breast cancer patients are diagnosed, they first undergo surgery to remove the tumor, then receive chemotherapy and radiation to kill any remaining cancer cells. Women whose tumors are estrogen receptor positive then receive tamoxifen for up to ten years.

The clinical problem addressed by this paper is the large minority of patients who experience cancer recurrence during or after tamoxifen treatment. We model this problem in lab by comparing tamoxifen sensitive breast cancer cells with cells selected for tamoxifen resistance. We then use these resistant cells to compare gene expression of metabolic enzymes. Pyruvate dehydrogenase is an important metabolic enzyme that converts pyruvate to acetyl CoA; links glycolysis to the tricarboxylic cycle (TCA cycle) and is regulated by phosphorylation. This phosphorylation is carried out by pyruvate dehydrogenase kinase 4 (PDK4).

During cellular metabolism, pyruvate is converted to acetyl coenzyme A through the pyruvate dehydrogenase complex. Pyruvate is important because it is produced in the cytoplasm through glycolysis and transported into the mitochondria. Decarboxylation catalyzed by pyruvate dehydrogenase generates acetyl CoA for use in the citric acid cycle. Pyruvate dehydrogenase, therefore, serves as an intermediate enzyme between the important metabolic pathways of glycolysis and the citric acid cycle. Since pyruvate is important in mitochondrial ATP generation, if there is a disruption in the pyruvate metabolism, it might result in a mutation in the enzymes that are involved in its regulation, thereby opening opportunities for diseases.

Pyruvate dehydrogenase is regulated by reversible phosphorylation by pyruvate dehydrogenase kinase (PDK) of three serine residues in the E1 subunit. (Gray, et al. 2014; Gang et al. 2014). This phosphorylation is facilitated by one of the four PDKs (PDK1, PDK2, PDK3, PDK4). PDK1 phosphorylates all the three (Ser^{232}, Ser^{293}, Ser^{300}) sites that can inhibit PDH activity. Meanwhile PDK2, PDK3 and PDK4 can only phosphorylate Ser^{293} and Ser^{300}. In Tamoxifen sensitive cells in the presence of PDK4, PDH activity is inhibited, but in tamoxifen sensitive cells there is high expression of PDK4 and high PDH activity. This suggests that there might be a phosphatase that dephosphorylates PDH after PDK4 phosphorylation, or that PDK4 activity is impaired in TAMR-MCF-7 cells.

The Skildum lab has completed research on the reason why estrogen receptor positive breast cancer cells become resistant to tamoxifen and where this resistance could possibly be taking place. Previous lab observations indicated that tamoxifen resistant cancer cells (TAMR-MCF-7) have increased expression of pyruvate dehydrogenase kinase-4 (PDK4), high pyruvate
dehydrogenase activity which is phosphorylated by PDK4, and increase mitochondrial DNA in relation to the parent cells which are not resistant to tamoxifen (MCF-7L) (Walter et al, 2015). The high expression of pyruvate dehydrogenase (PDH) led to the hypothesis of this research that, TAMR-MCF-7 have hypo-phosphorylated PDH. To test this hypothesis, the western blotting technique is used to detect proteins that have been separated by gel electrophoresis and transferred onto a membrane. This technique is used to identify specific proteins from a complex mixture of proteins from cells, by size and transferring to a membrane which the proteins are marked using primary and secondary antibodies. The detection in this experiment was carried out using two antibodies, anti-phospho PDHE1-AType 1 (ser293) and PDHE1alpha (Rardin et al, 2009).

Hypothesis
Tamoxifen Resistant cells have hypo-phosphorylated PDH.

Materials and Method

Part 1

The ATCC-MCF-7 cells were treated with glucose alone, galactose alone, glucose + FBS and galactose + FBS and cultured in an incubator. Glucose plus FBS is the cells’ normal growth media. Replacing glucose with galactose starves the cells for energy gained through glycolysis, while omitting FBS starves cells of hormonal signals. Proteins were isolated and separated through the SDS-PAGE.

12ul of each of the four proteins as well as 6ul of the molecular weight markers were loaded into the wells in the SDS-PAGE gel and ran for 5 minutes at 50 V. The voltage was later increased to 120V to fun for an hour.

The membrane was placed in a methanol for two minutes then placed in transfer buffer for 10-15 minutes. Two western blot filter papers and two foams were also equilibrated in the transfer buffer for 10-15 minutes. A sandwich of the gel and the membrane, filter papers and foams was created avoiding the formation of bubbles with the blot on the cathode and the gel on the anode. The sandwich cassette was then placed on an ice block in the transfer tank and kept to transfer in a cold room at a constant current of 100Amp for 90 minutes.

After the transfer, the membrane was blocked in Blotto buffer at room temperature for 30 minutes and incubated overnight in two primary antibodies (anti-phospho PDHE1-AType 1
(ser293) and alpha anti pyruvate dehydrogenase) diluted in Stringent antibody dilution buffer at 4 degrees. The blot was then rinsed 3 times with PBS-T for 5 minutes each and incubated in the Goat-anti-rabbit-HRP conjugated secondary antibody solution for an hour at room temperature. It was then washed with PBS-T 3 times for 5 minutes each. The next step was imaging and analyzing the data, which was done by applying the chemiluminescent substrate (1.5ml peroxide plus 1.5ml luminescent) to the blot and the signal was captured using X-ray film and quantitated by densitometry. The membrane was then washed with PBS-T 3 times and placed in actin to ensure equal loading of sample.

**Part 2**

Because of difficulty detecting PDH phosphorylation in breast cancer cells, we chose a cell line that has high PDH expression for use in optimizing western blotting conditions. Instead of the four treatments of the ATCC-MCF-7 cells as in the first part 1, just two treatments of a cardiac myocyte cell line cells used. One of the samples was treated with Dichloroacetate (DCA), a non-specific PDK inhibitor which should reduce PDH phosphorylation and serve as a negative control. Untreated cardiac myocytes were used as a positive control. In this experiment we also sonicated the lysates prior to blotting to increase the solubilization of mitochondrial proteins. The same experimental procedures were used as in part one except after the transfer, the membrane was blocked with 3-5% BSA and TBS-T at room temperature for 30 minutes and incubated overnight in two primary antibodies (anti-phospho PDHE1-AType 1 (ser293) and alpha anti pyruvate which were diluted with TBS. Instead of rinsing the membrane with PBS-T all the rinse were done with TBS-T.

**Results**

**Western Blot Images**

**Part 1**
Figure 1. ATCC-MCF-7l cell lysate were prepared in RIPA buffer with an addition of NaF. 12ul of each protein sample was separated by SDS-PAGE. The Protein samples were incubated in both PDH proteins we re anti-phospho PDHE1-AType1(ser293) and PDHE1alpha primary antibodies at 4 degrees overnight. Then we incubated the membrane in Goat-anti-rabbit-HRP conjugated secondary antibody solution for an hour at 4 degrees. Proteins were detected through applying chemiluminescent substrate to the blot and the signal was captured using X-ray film and quantitated by densitometry. Anti-phospho PDHE1-AType1(ser293) indicated no bands while PDHE1alpha indicated bands at predicted molecular weight (42kDa) (A). Actin indicated the presence of proteins though anti-phospho PDHE1-AType1(ser293) indicated no phosphorylation signal (B).
ATCC 16 cells lysate were prepared with an addition of Dichloroacetate (DCA) and sonicated. 12ul of each protein sample was separated by SDS-PAGE. The Protein samples were incubated in both PDH proteins were anti-phospho PDHE1-AType1(ser293) and PDHE1alpha primary antibodies at 4 degrees overnight. Then incubated in Goat-anti-rabbit-HRP conjugated secondary antibody solution for an hour at 4 degrees. Data analyzed through applying chemiluminescent substrate to the blot and the signal was captured using X-ray film and quantitated by densitometry. Anti-phospho PDHE1-AType1(ser293) and PDHE1alpha indicated bands at predicted molecular weight (42kDa). Actin indicated the presence of proteins and equal loading.

Since DCA reduces induced dephosphorization of E1α subunit of pyruvate dehydrogenase complex, the samples with DCA indicated no PDH phosphorylation while the treated samples indicate phosphorylation shown by the presence of the double band.

**Discussion and Future plans**

Conditions for the detection of PDH phosphorylation in cultured cells we identified during the two experiments mentioned above. The presence of phosphate in the blotting buffer interfered with the primary antibody interaction with the phosphor—PDH. After switching to Tris based buffers there was a significant increase the signal. In the next experiments we will use the cardiac
myocyte cells to optimize the western blotting conditions using Tris buffers, to reduce the background and increase the specific signal. Once conditions are optimized, we will compare PDH phosphorylation in tamoxifen sensitive versus tamoxifen resistant human breast cancer cells.

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References


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