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The Affects of Thiamine on NRF2 Expression in MCF7 ER+ Breast Cancer Cells

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Objective

Our project's objective is to investigate the effect of differing doses of thiamine treatments in ER+ breast cancer cell lines, specifically MCF7, and the effect on the expression of the transcription factor NRF2.

Abstract

Cancer metabolism and exploitation of metabolic pathways are a key focus in oncologic research, and has been for over 50 years. While many developments in identifying these specific pathways have been made, the complex nature of cancer metastasis and the many factors that promote and inhibit growth and survival of cancer cells makes this an enormous feat for researchers everywhere to fully uncover and understand. One particularly interesting pathway exploited by tumor cells is that of thiamine-dependent pathways. Our project's objective is to investigate the effect of differing doses of thiamine treatments in ER+ breast cancer cell lines, specifically MCF7 mentioned in this paper, and the effect on the expression of the transcription factor NRF2.

Introduction

Malignant cells often exploit metabolic pathways within normal cells to support their growth and reproduction. The reprogramming of these metabolic pathways allow for tumorigenesis and metastasis (DeBerardinis and Chandel, 2016). Identifying and understanding which pathways are being altered and promoting tumor growth is at the center of cancer research today. Of particular interest is the transformation of thiamine-dependent pathways by cancer cells (Zastre, et al. 2013).

Thiamine, commonly referred to as vitamin B1, is necessary for metabolizing carbohydrates into energy. In addition to pathways controlled by thiamine-dependent enzymes being altered by cancer cells (Liu, et al. 2014), thiamine deficiencies have been associated with

advanced cancers, and to resolve this, cancer patients often are prescribed with thiamine supplements (Boros et al 1998, Comín-Anduix, 2001). However, further studies have shown correlation with thiamine supplementation and tumor proliferation, and to further complicate the understanding of thiamine's role in cancer, treatment with high dosage thiamine has shown inhibitory effects on cancer cells (Comín-Anduix, 2001). These findings make it difficult to understand whether or not thiamine is beneficial or harmful to cancer patient's prognosis. If thiamine supplementation is prescribed to resolve thiamine deficiencies, but also promotes cancer cell metabolism, this could be detrimental to patient's outcome. On the other hand, if higher dose thiamine supplementation and treatment can be used to inhibit cancer promoting pathways, thiamine, although harmful in smaller doses, could be a promising therapy.

NRF2, a transcription factor found in the nucleus of cells, is an important protector against oxidative stressors. However, despite its protective properties, studies have shown NRF2 to play a role in cancer progression. Researchers believe that this is due to NRF2 also having a protective effect on cancer cells, and even allowing for chemo-resistance and tumorigenesis, and have also found elevated levels of NRF2 and upregulation of NRF2 dependent genes in cancer cells (Sporn and Liby, 2012). Another study of interest showed the knock-down of NRF2 blocked HIF-1a signaling and inhibited angiogenesis of colon cancer cells (Kim, et al., 2011). These studies and others like them show that NRF2 inhibition could be a promising target for cancer therapies and treatments.

Our project explores the possibility of using thiamine as a treatment to decrease NRF2 expression in cancer cells by using the ER+ breast cancer cell line MCF7 and administering differing concentrations of thiamine treatments. We hypothesize that increasing levels of thiamine dosage to 3T will have an inhibitory effect on NRF2, decreasing the expression in cancer cells.

By decreasing expression of NRF2, thiamine could be a viable treatment to target this and other pathways in cancer cells for future studies.

Materials and Methods

Cell Culture and Treatment

The estrogen-receptor-positive (ER+) breast cancer cells (MCF7 HC) were cultured and treated with varying thiamine treatments (0T, 0.3T, and 3T) by Hunter Connell, and then harvested for lysis and nuclear extraction.

Cell Lysis and Nuclear Extraction

First, the cell growth media was removed and the cell dishes were washed using cold PBS, which was then removed. Then cytoplasmic extract buffer containing 0.5% protease inhibitor cocktail (PI) and 1% PMSF was added to the dishes. Adherent cells were then scraped and collected into micro-centrifuge tubes, chilled, and 10% NP-40 was added to each tube (62.5 μ L/mL buffer). Afterward, the tubes were spun for 10 minutes in a centrifuge to form a nuclear pellet. The supernatant, which is the cytoplasmic extract fraction, was removed and saved for analysis. The nuclei pellet was then re-suspended in the nuclear extraction buffer and vortexed for 30 seconds every 10 minutes for 40 minutes and then spun in the centrifuge for 5 minutes. Finally, the supernatant, which is the nuclear extract fraction, was removed and transferred to a new tube for analysis.

Protein Quantitation Assay

After obtaining the nuclear and cytoplasmic extract fractions, a protein quantitation assay was performed. Each sample was diluted with a 5x dilution factor, then 10 μ L of each dilution and 10 μ L of 8 standards were aliquoted in triplicates to a 96 well plate. Next, 200 μ L of bicinchoninic acid (BCA) reagent was added to each well. The plate was then incubated for 30

minutes and placed in the spectrophotometer. SoftMax Pro software was used to measure absorbance and thus protein concentration in each sample.

Western Blot

Using the protein concentrations obtained from the protein quantification assay, calculations were done to ensure that each sample was brought to a 50 μ g total. The resulting amount of each sample, diluent, and loading buffer were added to micro-centrifuge tubes, boiled for 5 minutes and briefly centrifuged. Next, the gel electrophoresis running apparatus was set up, filled with running buffer, and the samples were loaded into a 12% agarose gel alongside the 5 μ L protein ladder. The apparatus was run on 110V until the lowest blue indicator line was run off the gel to ensure proper separation of the bands.

Next, a PVDF membrane was cut to gel-size and shaken in methanol for 10 minutes. Meanwhile, a transfer cassette was assembled containing 2 sponges and 2 gel-sized filter papers, one on each side of the cassette. The gel was removed from the running apparatus and the wells were removed. The membrane was placed directly onto the gel, a filter paper on top of that, and then a sponge on top of the filter paper. This was placed into the cassette with the same sponge-filter paper assembly on the other side of the gel. The cassette was then closed and placed into the transfer tank, which was then filled with transfer buffer. The tank was entirely surrounded and covered with ice. The transfer was run on 250A for approximately 2.5 hours.

Membrane Cutting and Antibodies

After the transfer was completed, the membrane was cut across the 55 kDa band so that actin (42MW), P84 (84MW), and NRF2 (MW72) could be imaged. The membranes were put into milk to block and shaken for 30 minutes at room temperature. They were then washed 3 times for 10 minutes each with TBST. Primary antibody was added to each respective blot (Actin

and NRF2 initially) and they were shaken and refrigerated overnight. The following day the membranes were washed 3 times with TBST for 10 minutes each and then 5mL of the appropriate secondary antibody was added to each blot and left on for an hour. Afterwards, the blots were again washed 3 times with TBST.

FluroChem Imaging

After washing off the secondary antibody with TBST, the membranes can be imaged. A 1:1 SuperSignal developing solution was made and added to membranes in a dish dropwise until the membrane was completely covered. After sitting for a few minutes, the dish with the membrane was placed in the imager and images were acquired using FluroChem software. Images can be seen in figures 1-3.

Results

Actin was imaged to be used as a cytoplasmic loading control (fig. 1). The bands, staying uniform throughout, shows that loading gels and the imaging process went without error. Actin has a molecular weight (MW) of 42 and is typically seen around 42 kDa as seen in figure 1. The first three lanes, from left to right are nuclear samples, and the last four lanes are cytoplasmic samples. The last lane shows the positive cytoplasmic control: (+) MG132.

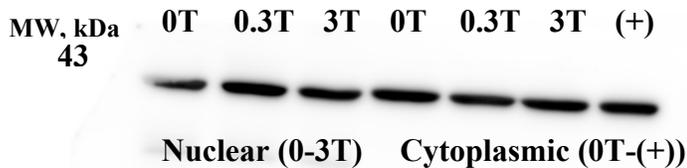


Figure 1: Western blot analysis of actin expression in MCF7 breast cancer cells treated with increasing doses of thiamine (0T-3T). Expression is seen around 43 kDa.

P84, found in the nucleus, is a “house-keeping” gene and was imaged to be used as a nuclear loading control (fig. 2). As seen in figure 2, the bands stay fairly constant so changes in

NRF2 expression can be attributed to the thiamine treatments. Three bands are seen at 84 kDa, the MW of P84. The bands from left to right show increasing thiamine concentrations, 0T, 0.3T, and 3T.

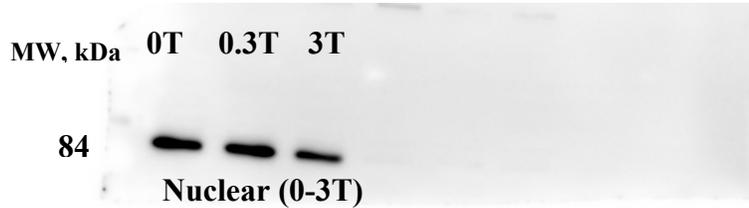


Figure 3: Western blot analysis of P84 expression (around 84 kDa) in MCF7 breast cancer cells treated with increasing doses of thiamine. Pictures are three nuclear samples with increasing thiamine dosage from left to right.

NRF2 expression is seen in figure 3 around 130 kDa. Although it is listed in some literature that NRF2 has a molecular weight, more recent studies and companies selling NRF2 antibodies have said NRF2 expression is more frequently seen between 95-130 kDa (Lau, 2013). As such, we proceeded with our study with the bands seen at 130 kDa in figure 3. Densitometry was performed on both the P84 and NRF2 blots using these bands.

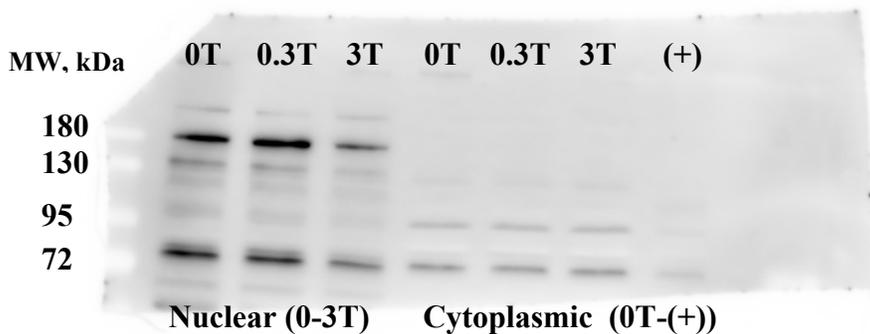


Figure 3: Western blot analysis of NRF2 expression (around 130 kDa) in MCF7 breast cancer cells treated with increasing doses of thiamine. The first three lanes, from left to right are nuclear samples, and the last four lanes are cytoplasmic samples.

We obtained the densitometry measures for NRF2 and then P84, divided the NRF2 measurement by the P84 values and normalized them to the 0T control, which we set equal to 1,

to show if increasing thiamine concentration in treatments affects the expression of NRF2.

Figure 4, the densitometry analysis shows a 50% decrease with increased thiamine concentration in the cell treatments.

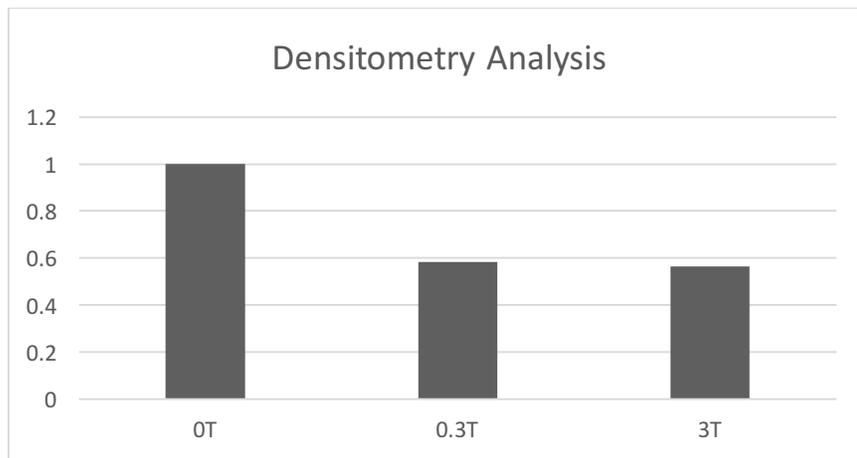


Figure 4: Densitometry analysis of NRF2 expression normalized to P84. The expression of NRF2 decreases by about 50% with increased thiamine concentration.

Discussion:

Thiamine and NRF2 have both individually been of key focus in cancer research, but not until now have they been considered together as a viable treatment pathway against tumor cells. NRF2 has previously been proven to play a role in having protective effects over cancer cells and causing chemo-resistance due to its antioxidant protective properties that are exploited in cancer as well (Sporn and Liby, 2012). It is also known that cancer cells exploit metabolic pathways to support their growth and survival and to meet their nutrient and energy needs to maintain tumorigenesis and proliferation (Zastre, 2013). Because of this, vitamin supplementation, specifically thiamine (vitamin B1), although needed because of thiamine deficiencies found in cancer patients, can be more harmful than beneficial to patients. However, while these smaller doses of thiamine may be harmful and promote cancer proliferation, it has been shown that high doses of thiamine have antagonistic effects on cancer cells (Lu'o'ng, 2013). These two areas of

previous research led us to explore the relationship between thiamine and NRF2. By treating cells with different concentrations of thiamine and measuring the effects using nuclear extraction and western blotting techniques, we were able to show a decrease in NRF2 expression with increasing thiamine concentration in the cell treatments as shown in both figures 3 and 4. This supports our hypothesis that high dosage thiamine treatments will inhibit the expression of NRF2. Densitometry (fig. 4) was performed to ensure that changes in NRF2 expression were due to the applied thiamine treatments, and not due to any outstanding factors in loading controls or otherwise.

Although there is literature supporting the higher expression of NRF2 than the commonly used 72 kDa (Lau), this initially set a limitation on our study before investigating why our blots consistently showed NRF2 at a higher band than expected. After switching our antibody to one whose company, GeneTex, confirmed the higher band expression around 130 kDa in nuclear samples, we could more confidently move forward with deciphering which bands on our blots were NRF2 (fig 3). Additionally, the known boundaries of thiamine treatments that cause inhibitory effects on NRF2 expression and tumorigenesis are not known, and our study was limited to testing a certain scale of treatments, 0T, 0.3T, and 3T concentrations. It would be of interest to further study and to quantify the exact boundaries and concentrations of thiamine that inhibit NRF2 and tumorigenesis, as well as quantify the lower boundary of the spectrum: thiamine concentrations that promotes cancer growth. Additionally, it would be helpful for future projects to submit their data showing the higher expression of NRF2 so that antibody companies will unanimously change this in their data as to prevent further confusion among scientists.

Conclusion

The shown decrease in expression of NRF2 in the MCF7 ER+ breast cancer cell line shows promising evidence for high dosage thiamine treatments as a viable cancer therapy to

prevent proliferation of cancer cells. Our study showed that expression of NRF2, a known protagonist in cancers, is inhibited with greater concentration of thiamine in applied treatments. The seemingly fine balance between cancer patient's need for thiamine supplementation due to known thiamine deficiencies in advanced stage cancers, the exploitation of thiamine-dependent pathways in cancer cell metabolism and proliferation, and the high thiamine dosage treatments to inhibit cancer cell growth and metastasis is one to be further studied to find the exact values and boundaries of thiamine's role in cancer metabolism and inhibition, and what other, if any, pathways can be manipulated by thiamine treatments.

References

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