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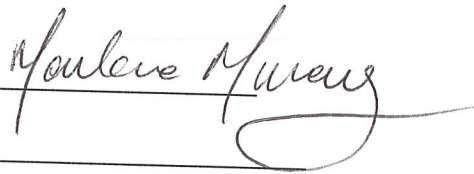
The Effects of Omega-3 Fatty Acids on Intracellular Inositol Levels in *Saccharomyces Cerevisiae*

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March 28, 2016

Advisor: Marlene Murray

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Department: _____ Biology _____

Abstract

Bipolar disorder is a severe and chronic debilitating mental disorder characterized by extreme mood swings between mania and depression. The current medications for bipolar disorder, lithium and valproate, have been associated with numerous negative side effects. Although the therapeutic mechanism by which lithium and valproate (VPA) exert their effect is unknown, a leading hypothesis implicates inositol depletion as a mechanism of action. On the other hand, omega-3-fatty acids have been shown to relieve symptoms of bipolar disorder. In this study, we compare the effects of VPA to the effects of decosahexaenoic acid (DHA) on *Saccharomyces cerevisiae* growth and intracellular inositol concentration. Intracellular inositol levels were examined using a modified enzymatic assay for inositol, which correlates light absorbance to intracellular inositol concentration. The results show that similar to valproate, DHA inhibits cell growth. In addition, unlike valproate, DHA does not decrease intracellular inositol levels.

Introduction

Bipolar disorder is a chronic and severe mood disorder affecting 1-3% of the United States population. There are many subtypes of bipolar disorder; however, the defining feature of the disorder is the presence of two very distinct and extreme mood phases: the manic phase and the depressive phase. Manic phase, as the term implies, is characterized by feelings of heightened energy, euphoria, and hyperactivity. Some physical symptoms of the manic phase are disorganized speech, racing thought, rapid bursts of speeches, and little need for sleep. However, the psychological effects of the manic phase include but are not limited to: overconfidence, delusions, recklessness, and impulsiveness, and impaired judgment. Thus, the patient is more

likely to engage in dangerous and high-risk behaviors during the manic phase. On the other hand, the depressive phase is characterized by feelings of extreme hopelessness, worthlessness, sadness, and inability to feel pleasure. These symptoms can be manifested through fatigue, lack of motivation, significant appetite and weight changes, sleep problems, and even suicidal thoughts. During the depressive phase, the National Institute of Mental Health has found that as many as one in every five people with bipolar disorder commits suicide (National Institute of Mental Health, 2016).

Not only is bipolar disorder fairly lethal, it is also not rare. According to the National Institute of Health, bipolar disorder can affect up to 5.7 million American adults, or 2.6% of the American population aged 18 and older, in a given year (National Institute of Mental Health, 2016). Yet even though bipolar disorder affects a significant number of the American population, the “perfect” cure for the disorder is yet to be discovered. Currently, the primary medications used for treating the bipolar disorder are valproic acid or valproate and lithium. Unfortunately, lithium and valproate are not always effective (Vacheron-Trystram et al., 2004). Not only can they be inconsistent in their ability to stabilize moods, but they are also associated with numerous negative side effects including muscle pain, acne, nausea, liver failure, and even more suicidal thoughts and behaviors (Young and Hammond, 2007). Unfortunately, the therapeutic mechanism of lithium and valproate currently remains unknown, further complicating the generation of a better mood stabilizer. However, one way to discover the therapeutic mechanism is to focus on their common target of action. Currently, inositol has been focused on as the common target of lithium and valproate.

Inositol, a cyclohexane with six alcohol groups, is a biological molecule that is used widely throughout the body. One of the reasons for its prevalence is that it is a precursor of two

very important second messengers in the body: inositol triphosphate (IP₃) and diacylglycerol (DAG). Both IP₃ and DAG are involved in activating the second messenger system in circulatory, digestive, reproductive, nervous, and respiratory systems. Thus, because of inositol's extensive function in the body, it is easy to see how inositol would be implicated in a neurological disorder such as bipolar disorder. Lithium and valproate inhibit steps in the metabolic synthesis and breakdown of inositol so that the less inositol or products of inositol are produced. Lithium inhibits inositol monophosphatase (IMAP), which converts inositol 1-phosphate, a precursor of inositol, to inositol (Meyer et al., 2011). On the other hand, valproate inhibits the conversion of glucose 6-phosphate to inositol 1-phosphate by inhibiting inositol 1-phosphate synthase (Deranieh et al., 2013). Valproate also suppresses the action of PKC, which is one of the key enzymes activated by DAG, a product of inositol cleavage (Chen et al., 1994). Because inositol depletion is a common effect of both types of drugs, inositol depletion hypothesis is the currently leading hypothesis as a possible mechanism through which these mood stabilizers exert their effects.

Recently, omega-3 fatty acids have been emerging as a possible source of treatment for bipolar disorder. Omega-3 fatty acids are polyunsaturated fatty acids that are essential for humans. They play an especially crucial role in brain function and development as omega-3 fatty acids are used to make myelin sheaths of the neurons. Examples of omega-3 fatty acids include docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA). They are commonly found in fish oil, egg oil, flaxseed, and walnuts. Omega-3 fatty acids have been shown to be effective in treating several diseases including heart disease, diabetes, rheumatoid arthritis, osteoporosis, depression, schizophrenia, attention deficit hyperactivity disorder, and bipolar disorder (National Institutes of Health, 2016). Various double-blind, placebo-controlled studies have shown that

omega-3 fatty acids help in lessening the symptoms of bipolar disorder in bipolar patients (Stoll et al., 1999). Although more studies are needed to definitively secure omega-3 fatty acids as an effective treatment for bipolar disorder, the results of the various experiments seem promising. The use of omega-3 fatty acids holds several advantages over the use of lithium and valproate. The most significant advantage is that the use of omega-3 fatty acids has a potential to produce less side effects because omega-3 fatty acids are already needed and beneficial for the body.

Our research seeks to compare the effects of omega-3 fatty acids to the effects of valproate, focusing specifically on their effects on cell growth and intracellular inositol levels. The overarching goal is to discover more about the therapeutic pathway of omega-3 fatty acids and thus contribute to the possible establishment of omega-3 fatty acids as bipolar disorder treatment. Using *Saccharomyces cerevisiae*, which is more commonly known as baker's yeast, we sought to determine the effect of DHA on cell growth and intracellular inositol levels. Inositol levels were quantified by carrying out a spectrophotometric assay that correlates the inositol concentration with light absorbance. *S. cerevisiae* is a suitable research subject because although it is unicellular, it is eukaryotic and thus shares similar cellular and metabolic processes as human cells. The spectrophotometric assay relies on a coupled reaction in which color is produced as iodonitrotetrazolium (INT) is converted into formazan by diaphorase while myo-Inositol is converted to myo-Inosose by myo-Inositol dehydrogenase (MIDH) with the reduction of NAD^+ to NADH (Figure 1). Inositol had to be coupled to a conversion of INT to formazan because inositol is colorless and thus cannot give any spectrophotometric reading. However, formazan produces a red color that deepens as the concentration increases. Since inositol conversion is linked to INT conversion, inositol concentration can be linked to the product of the INT conversion, which is formazan.

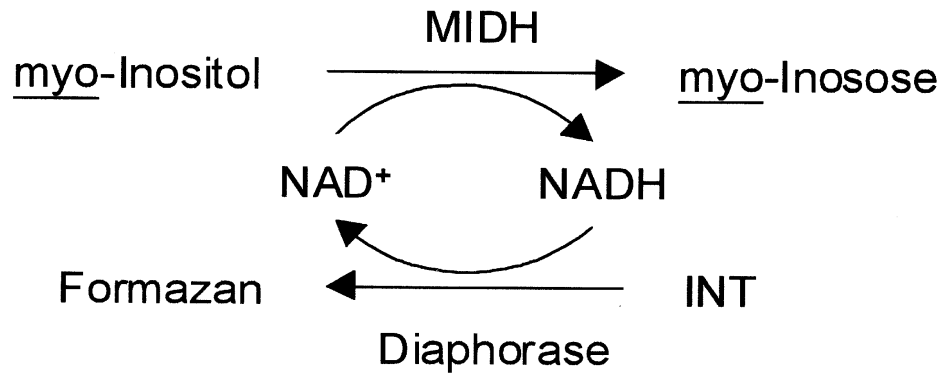


Figure 1. Coupled reaction in which myo-Inositol is converted to myo-Inosose by MIDH and INT is converted to formazan by diaphorase, producing a visible color.

Method

Cell Growth

Yeast cells that had been stored at -80°C were taken out and plated onto YPD plates. YPD contains 2% glucose, 2% bacto-peptone, 2% agarose and 1% yeast extract. The cells were then allowed to grow for 4 days. 100 ml of complete media (0.069% vitamin-free yeast base, 2% glucose, 0.201% ammonium sulfate, 20mg/l adenine, 20mg/l arginine, 10mg/l histidine, 60mg/l leucine, 20mg/l lysine, 20mg/l methionine, 300mg/l threonine, 20mg/l tryptophan, 40mg/l uracil, and vitamins as described in Culbertson and Henry) was inoculated with cells from these plates and grown for 24 hours.

In the meantime, four types of media were generated. 2000 mL of the complete media were divided into four 500 mL flasks, each labeled control, 2.5 mM VPA, 0.4 mM DHA, and 0.8 mM DHA. Into the 2.5 mM VPA flask, 0.4 mM and 0.8 mM DHA flasks, appropriate amounts of VPA and DHA were added.

After the 24 hour growth of the yeast cells, the optical density of the cells was measured at 550 nm after being diluted tenfold. How much cells to be put into each of the 500 mL culture was determined based on having the final concentration to be 0.1 M using $C_1V_1 = C_2V_2$.

Appropriate amounts of cells were put into each of the four flasks and grown for 24 hours in a shaking incubator at 150 rpm. One ml from each media were serially diluted five fold then plated again onto YPD plates and allowed to grow for 24 hours. Single colonies were determined by growth on YPD plates.

Obtaining Cell Extracts

The remaining experimental cultures were used to make cell extracts to be used for the enzymatic assay. The cultures were aliquoted into centrifuge tubes and centrifuged until all of the supernatant was discarded and only cell pellets remained. The pellets of each culture were weighed. To determine the amount of glass beads to be added, about half the volume of the weighed cell pellets was approximated. After the glass beads were added to the pellets, the mixture was vortexed. The vortex at high speed broke open the cells. Cells were vortexed at 2 minute intervals on ice. The resulting cell extracts were stored at -80 °C.

Standard Curve Generation

Known concentrations of inositol samples were made by mixing a powder form of stock inositol with appropriate amounts of water. The concentrations used were: 0 nM as a control, 5 nM, 10 nM, 25 nM, 50 nM, 100 nM, and a 1000 nM. The inositol samples were deproteinized with 16% hydrochloric acid by mixing 250 µl of the sample with 250 µl of the acid and being centrifuged at 5000 x g for 10 minutes. The supernatants were removed and neutralized with 2.0 M K₂CO₃. Each sample was neutralized individually with approximately 130-150 µl of K₂CO₃. Neutralization was confirmed using pH test strips. After neutralization, the samples were centrifuged again at the same speed. After centrifugation, 100 µl of the supernatant was added to

10 µl of premade hexokinase reagent (200 mM Tris-HCl buffer, 400 nM adenosine triphosphate disodium, 115 U/ml hexokinase). Hexokinase was added to ensure that there was no glucose left in the samples or the cell extracts as high glucose increases background that would interfere with the absorbance. The mixture was incubated at 37°C for 90 minutes and then heated in boiling water for 3 minutes. 20 µl of 4.5 M HCl was added to ensure all endogenous NADH and NADPH were eliminated. After 10 minutes, 3.0 M K₂CO₃ was added for neutralization. Again, neutralization was confirmed using pH strips. After neutralization 100 µl of each sample was mixed with 100 µl of the MI reagent (210 mM triethanolamine hydrochloride-32 mM K₂HPO₄ – KOH buffer (pH 8.6), 1.2% Triton X-100 (v/v), 10 mM β -NAD, 1.0 U/ml diaphorase, 0.1% (w/v) bovine serum albumin, 60 µg/ml INT). The samples were loaded onto a microplate and their absorbance was measured at 492 nm using a spectrophotometer. Then, 10 µl of myo-inositol dehydrogenase (MIDH) was added to start the reaction. The reaction was allowed to progress for 20 minutes and the absorbance was measured again at 492 nm. The change in absorbance from the reaction was plotted against the known concentrations of inositol and a straight-line graph was generated.

Intracellular Inositol Concentration

The same procedure used in generating the standard curve was repeated with the actual cells. Cells grown in the three types of media were measured: cells grown in control media without valproate or DHA, cells grown in 2.5 mM VPA, and cells grown in 0.4 mM DHA. The concentrations of VPA and DHA were chosen based on previous research that showed that 2.5 mM and 0.4 mM were the lowest possible concentration shown to produce inhibition of growth. The spectrophotometric results were correlated back to intracellular concentration using the previously generated standard curve. The protein present in the three types of media was

standardized with Bradford protein assay to ensure the standardization of amount of cells present in each type of media (He, 2011). The significance of the results was shown using a one-way ANOVA.

Results

Cell Growth

Control	215
VPA (2.5mM)	77
DHA (0.4mM)	127
DHA (0.8mM)	19

Table 1. A table recording the average cell growth numbers obtained in each type of plate. VPA and DHA both inhibit cell growth. 0.8 mM DHA is the most potent inhibitor.

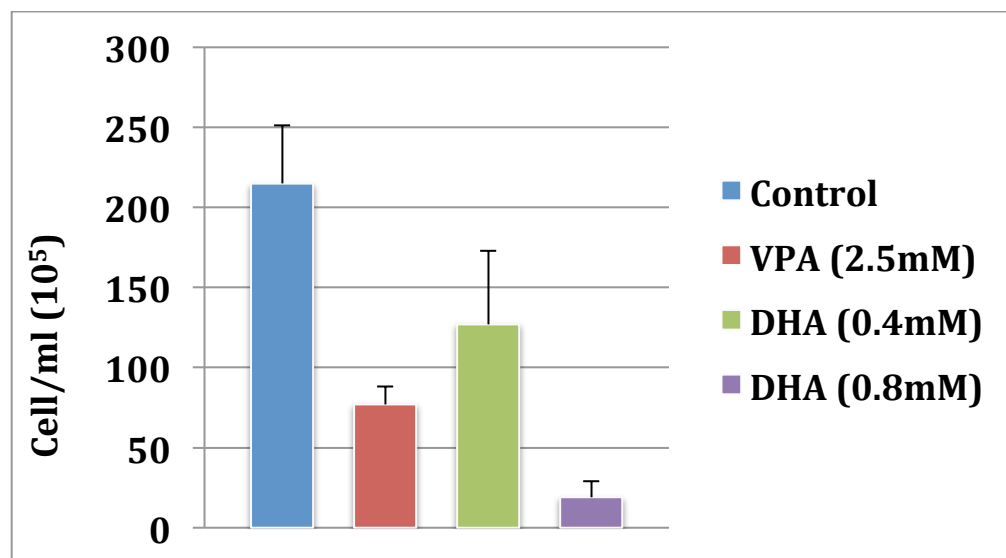


Figure 2. Average of cells grown for 24 hours in the presence and absence (control, n=11) of DHA (0.4mM, n= 10 and 0.8mM, n= 6) or VPA, n=11. $p = .005$ by ANOVA.

Standard Curve

Six assays using known concentrations of inositol were conducted to generate the standard curve. The combined results of the six assays were averaged to give an average absorbance for each concentration of inositol. As expected, with increased concentration of inositol, light absorbance increased. Using the averaged values, a straight-line graph was generated.

	Average
0 mM	0
1 mM	0.1675555
5 mM	0.45605
10 mM	0.87988667
25 mM	1.125555
50 mM	1.45127667
100 mM	1.49777667
1 M	1.69433333

Table 2. A table recording the average spectrophotometric values obtained for the six standard curve assays. The values indicate the light absorbance taken at 492 nm of each inositol concentration sample. A direct relationship between absorbance and inositol concentration is seen.

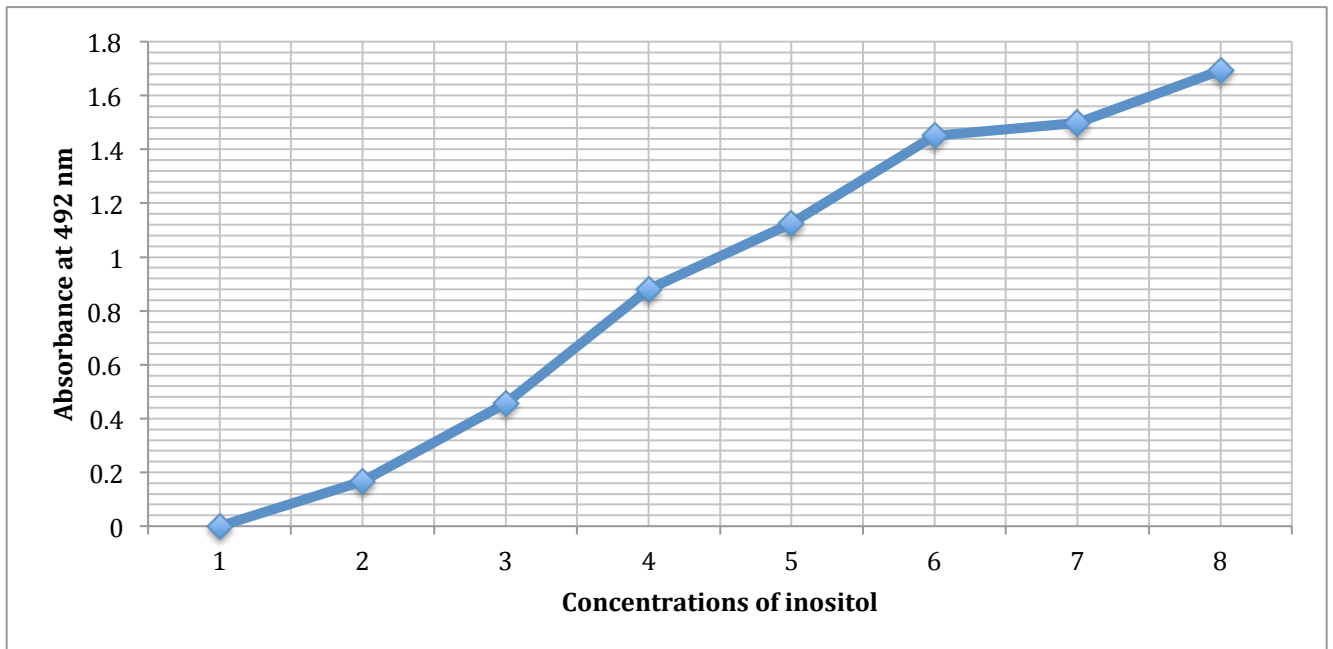


Figure 3. A straight line graph from the average values of each inositol concentration. The numbers 1-8 correspond to concentrations of inositol: 0 mM, 1 mM, 5 mM, 10 mM, 25 mM, 50 mM, 100 mM, and 1M, respectively.

Cell Extracts

	Average
Control	0.152
VPA (2.5mM)	0.0294
DHA (0.4mM)	0.394

Table 3. A table recording the average spectrophotometric values obtained for the three cell extracts. The values indicate the light absorbance taken at 492 nm of each inositol concentration sample.

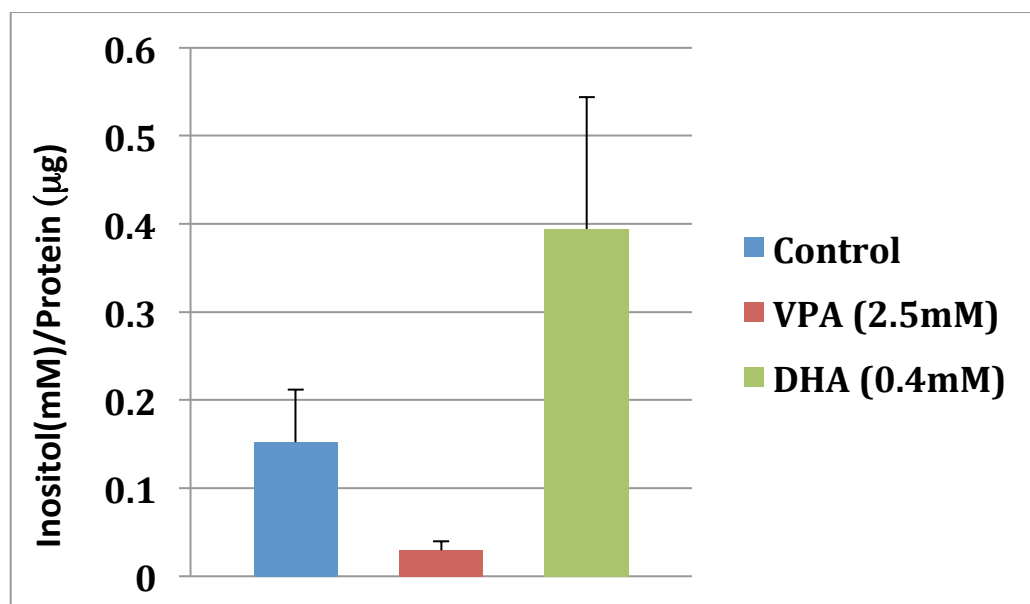


Figure 4. Extracts of cells grown in the presence and absence (control, n= 5) of DHA (n= 4) and VPA (n= 6) were assayed for inositol. $p= 0.018$ by ANOVA.

The cells grown in control, VPA, and DHA, were analyzed with 5, 4, and 6 assays, respectively. All the data was compiled and averaged to give the bar graph presented above.

Discussion

As can be seen, DHA inhibits the growth of *Saccharomyces cerevisiae*, an effect similar to that of the anti-bipolar drug VPA (Wang et al., 2013). Greater inhibition was seen in 0.8 mM DHA than in 0.4 mM DHA, possibly suggesting a stronger potency of 0.8 mM DHA than 2.5

mM VPA. Although VPA as expected lowered intracellular inositol levels, 0.4 mM DHA increased intracellular inositol levels. There are several possible explanations for this dissimilarity. One explanation is simply that the concentration of DHA was not high enough at 0.4 mM. As can be seen, the concentration of VPA used was much higher than that of DHA. It is possible that a 0.8 mM concentration of DHA will decrease inositol concentration, which is currently where the research is at. One assay testing 0.8 mM was run and it decreased intracellular inositol values more than was decreased by VPA. Further research and more runs are needed to corroborate the finding. Another explanation is that omega-3 fatty acids may mainly act to stabilize the depressive symptoms rather than both mania and depressive. Not only have omega-3 fatty acids been associated with treating major depressive disorder, some research indicates that it may be more potent in treating the depressive phase of bipolar disorder (Grosso et al., 2014). Since BD is characterized by a depressive stage followed by a manic stage and omega-3 fatty acids have directly been shown to be effective in only depressive stage as of now, difference between the effects of VPA and DHA may be expected since lithium and valproate work to stabilize both mania and depressive phases. Areas of future work would include increasing the concentration of DHA to compare its effect on inositol to that of VPA and running the same experiment with EPA, another omega-3 fatty acid.

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