IMPACT OF WESTERN DIET-INDUCED OBESITY ON ENDOCANNABINOID BIOSYNTHESIS IN MOUSE PANCREAS

By

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A capstone project submitted for
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APPROVED

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Abstract

Obesity incidence in the United States has increased dramatically in the past several years largely due to the overconsumption of foods rich in fats and sugars (i.e., the Western Diet). Obesity has been linked to a higher risk of developing other metabolic diseases such as Type-2 Diabetes. The endocrine pancreas is an important organ responsible for regulating blood glucose levels through the release of insulin; this becomes dysregulated in obesity with obese humans having high levels of circulating insulin. The endocannabinoid system is directly involved in regulating food intake and energy homeostasis. A complete endocannabinoid system has been located within the endocrine pancreas which may play a role in regulating insulin secretion following a meal. Previous research from our laboratory shows an increase mRNA expression of the biosynthetic enzyme diacylglycerol-lipase (DAGL) mRNA levels paired with a decrease in mRNA expression of the degradation enzyme monoacylglycerol-lipase (MGL) in obese mice. To test whether these mRNA changes are functionally relevant, we will use functional biochemical assays coupled with mass-spectrometry techniques to determine the extent to which enzymatic activity is involved with regulating insulin secretion. We hypothesize the enzymatic activity of these enzymes to reflect the same trend that their mRNA expression displayed. Any changes in enzymatic activity during obesity may have implications in insulin secretion during diet induced obesity.

Acknowledgements

I would like to give a special thanks to Dr. Nicholas DiPatrizio for making this opportunity possible and allowing me in taking part of your research team since joining the lab my second year. Moreover, a very special thank you to all of the DiPatrizio lab graduate students that have helped me along the way: Donovan Argueta, Pedro Perez, Mark Wiley, Bryant Avalos Leyva, and Courtney Wood. It is an honor to learn from these mentors and assist them in their own projects as well. I am very grateful that I was able to gain the knowledge of the techniques and methods for my capstone project as well.

I would also like to give recognition to the UCR University Honors Program for allowing me to contribute to the scientific research aspect of education by making the capstone project possible and extending me a seat in the Honors program at UCR**.** I will use the skills I have learned from the DiPatrizio lab inside and outside of the research lab as I pursue my interests in becoming a physician.

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Introduction

Over 42% of adults above the age of 20 are considered to be obese in the U.S. population. As the past data shows, this trend has been increasing by roughly 3% every year (CDC Data Briefs 2020). The prevalence and consumption of a lipid and carbohydrate rich Western diet is largely responsible for these obesity rates. Over a course of time, a diet like this leads to obesity which eventually increases one's risk for many other diseases like Type 2 Diabetes. The endocannabinoid system is an important regulator for the body's maintenance of homeostatic needs and metabolic balance. It functions under a signaling complex between a lipid-derived signaling messenger, an endocannabinoid, and its corresponding cannabinoid receptors, known as CB1 and CB2 (DiPatrizio 2012).

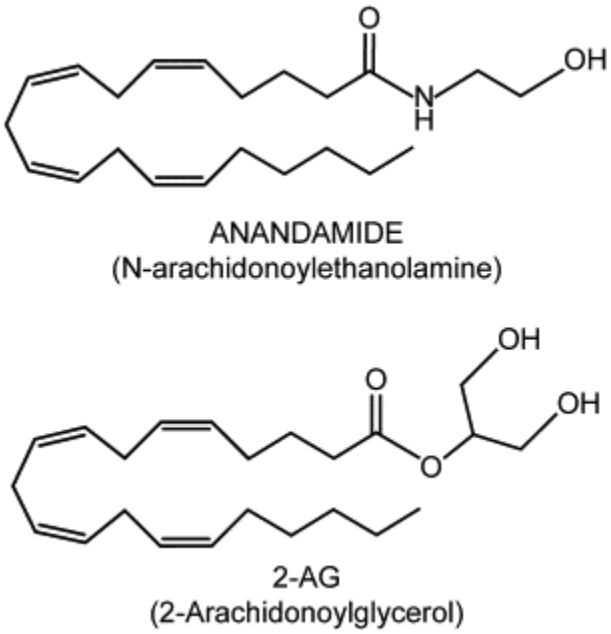

Figure 1. AEA (Anandamide) and 2-AG (2-Arachidonoylglycerol) Structure. As seen, both endocannabinoids are lipid derived ligands that produce signaling effects through cannabinoid receptors, CB1 and CB2.

The endocannabinoid system also includes endogenously produced lipid derived ligands 2-AG (2-arachidonoylglycerol) and AEA (anandamide), and the biosynthetic machinery responsible for biosynthesis and degradation of the endocannabinoids. One of the most studied and abundant endocannabinoids is, 2-AG. This endocannabinoid is synthesized using the enzyme diacylglycerol lipase (DAGL) and is degraded using the enzyme monoacylglycerol lipase (MAGL) (DiPatrizio 2012).

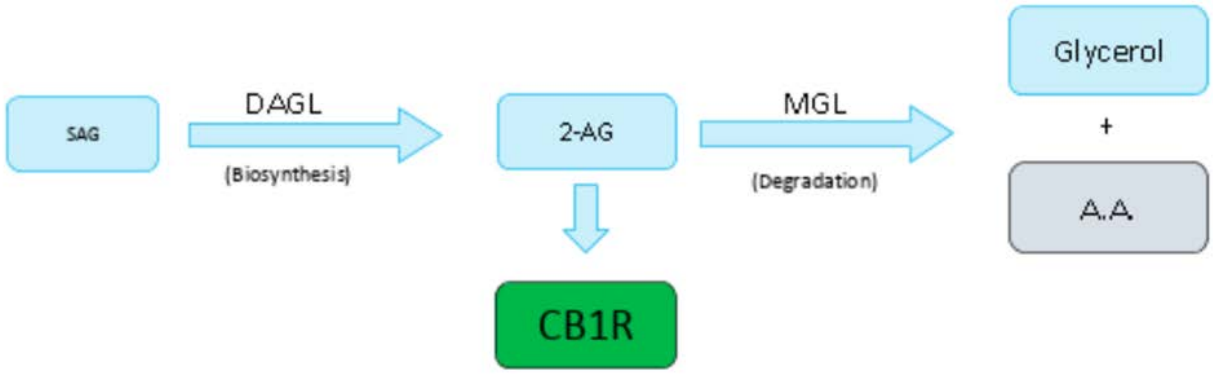

Figure 2. Endocannabinoid metabolism pathway. Stearoylarachidonoylglycerol (SAG) is synthesized into 2- arachidonoylglycerol (2-AG) using diacylglycerol lipase (DAGL). 2-AG then can be used for receptor signaling via cannabinoid receptor 1 (CB1). 2-AG is then degraded using monoacylglycerol lipase (MAGL).

Following Western diet intake, studies show that the endocannabinoid system becomes overactive in various organs, especially in the gut, where homeostatic levels of endocannabinoids are elevated. This has led to an increase in signaling, which may cause compulsive eating behaviors and the suppression of satiety, leading to diet-induced obesity (DiPatrizio 2012). Recent studies suggest that the endocannabinoid system plays a role in pancreatic β-cells in promoting insulin release, however, the mechanism is not well understood (Di Marzo 2008). Overall, this hyperactivation of the endocannabinoid system in conjunction with a fat-rich diet

has led to insulin-resistance, eventually leading to Type 2 Diabetes (Di Marzo 2008). Therefore, studies have been conducted, in a rodent model of diet-induced obesity, to investigate the changes of the endocannabinoid system in the pancreas in response to a Western diet.

A study was conducted in our lab in which mice were placed under a Standard chow diet or a Western diet. It was discovered that the mRNA expression of the biosynthetic enzyme, DAGL, increased in Western diet mice (Sanchez 2018). While the mRNA expression of the degradative enzyme, MAGL, decreased in Western diet mice (Sanchez 2018).

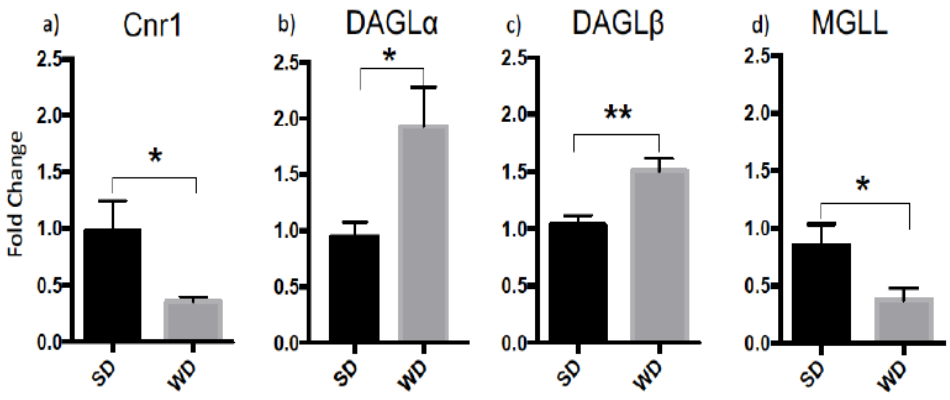

Figure 3. mRNA levels of the following enzymes were quantified using RT-PCR. Cnr1 and MGL expression levels show a decrease, while DAGL α/β expression levels increased after 60-day Western diet exposure. Data sets analyzed using two-tailed student t-test. **=$p<0.01$ *=$p<0.05$, n=3-4 (Sanchez 2018).

Additionally, there was no change in endocannabinoid levels in mouse pancreas in response to the Western diet (Sanchez 2018).

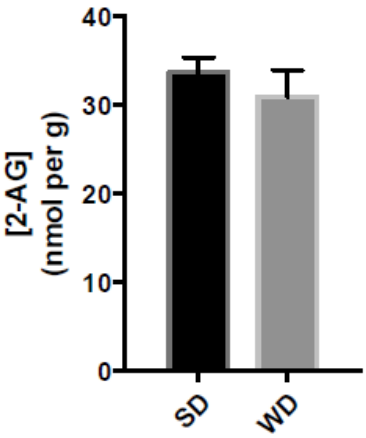

Figure 4. 2-AG levels did not change in mice pancreas in response to prolonged feeding of a Western diet. Data sets analyzed using two-tailed student t-test, ns= $p>0.05$, $n=10$ (Sanchez 2018).

These conclusions led us to study the possibility as to why endocannabinoid levels remained constant even though there are differences in biosynthetic and degradative enzyme mRNA expression. Therefore, this study investigates the enzymatic activity of the biosynthetic and degradative enzymes in order to further explore the stable levels of endocannabinoids between the two diets.

Methodology

Mice and tissue recovery: Mice were kept in feeding cages for 60-days in the vivarium. Each set of mice were kept on their respective diets and were allowed free access to either a Standard chow diet or the Western diet, set to mimic the nutritional intake of common diets in America (high fat/high sugar). Mice were monitored, and body weights were recorded three times a week for the 60-day period. After 60-days, the mice were euthanized and harvested where the pancreas tissue was removed and stored in the -80°C for further analysis. The protocols for animal care use were approved by the Institutional Animal Care and Use Committee at University of California, Riverside and were prepared according to the guidelines from National Institutes of Health.

Functional enzyme assay of MGL activity. Pancreatic tissues were homogenized, and protein concentration was quantified using a BCA protein assay normalized to 1mg/ml in a sucrose buffer. A substrate solution was prepared for a 19:2 MAG/500 µl reaction by adding 10mg Fatty-Acid-free BSA and 16ml of 50mM Tris-HCl pH 8.0 dropwise. During a 60-minute sonication (37°C), 100µl of normalized protein was added followed by 400 µl of the substrate solution. Samples were then incubated at 37°C then immediately placed into an ice bath. Samples were then prepared for lipid extract and LCMS analysis.

Lipid Extraction: Homogenized pancreatic tissue in 1.0 ml methanol solution containing internal standards. Then, lipids were extracted using chloroform alongside a water wash. Solutions were then separated by an open-bed silica gel column chromatography method and organic phases were collected. Eluates were gently dried under a N_2 gaseous stream at 37°C. Once dried, the eluate was resuspended in 100 µl 9:1 methanol-chloroform mixture and placed

with 1-microliter injection for ultra-performance liquid chromatography-tandem mass spectrometry analysis.

Results

Throughout the timeline, C57BL/J mice were placed on a 60-day Standard diet or a Western diet, respectively. Pancreatic tissue was then harvested and stored in the -80°C for further analysis. However, in order to optimize enzyme activity assays for pancreatic tissue, spare pancreatic tissue samples from other ongoing projects were used to conduct practice experiments. The following data has been recorded and analyzed from the practice experiments.

Sample #	Total Protein Concentration (µg)	Buffer Volume (µl)	Protein Volume (µl)	Final Concentration (µg/µl)
1	2253.236	556.19	443.81	1000
2	2394.794	582.43	417.57	1000

Table 1. Data table with values to normalize protein concentration between samples. Sample 1 (pancreatic tissue) was the experimental sample on the "new diet" being tested. Sample 2 was a pancreatic tissue of mouse on the control diet.

Sample #	Dilution (µl)	Sample #	Dilution (µl)	Blanks
1	6.25	2	25	Protein Blank
2	6.25	1	50	Substrate Blank
1	12.5	2	50	Control Blank
2	12.5	1	100	
1	25	2	100	

Table 2. Table displays the serial dilutions conducted in order to formulate the protein concentration curve for the MGL enzyme assay. Each sample was conducted in duplicate to increase sample size and minimize error.

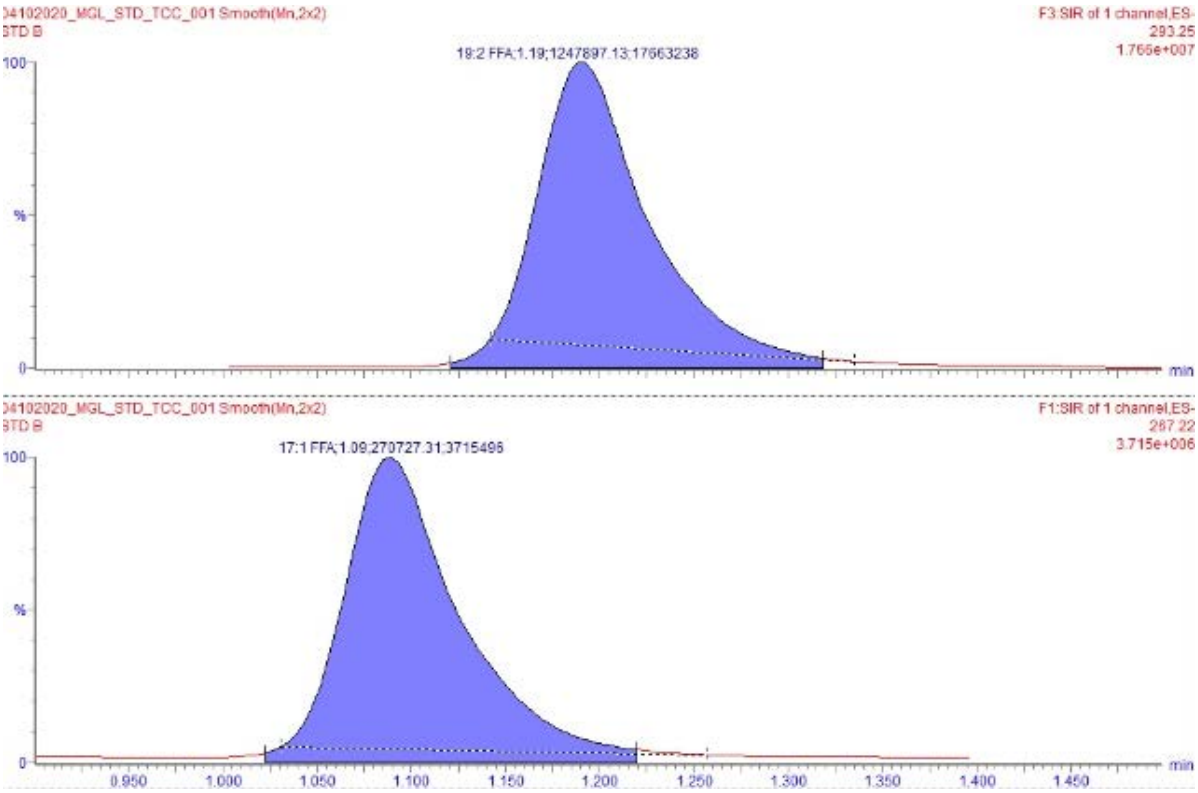

Figure 5. HPLC Chromatogram. Internal Standards (17:1 FFA) show a consistent and similar retention time. This chromatogram allows for the response ratios to be normalized and provides insight into product recovery differences. 19:2 FFA would be a product of 2-AG being degraded by MAGL. This method allows us to test for variability between samples.

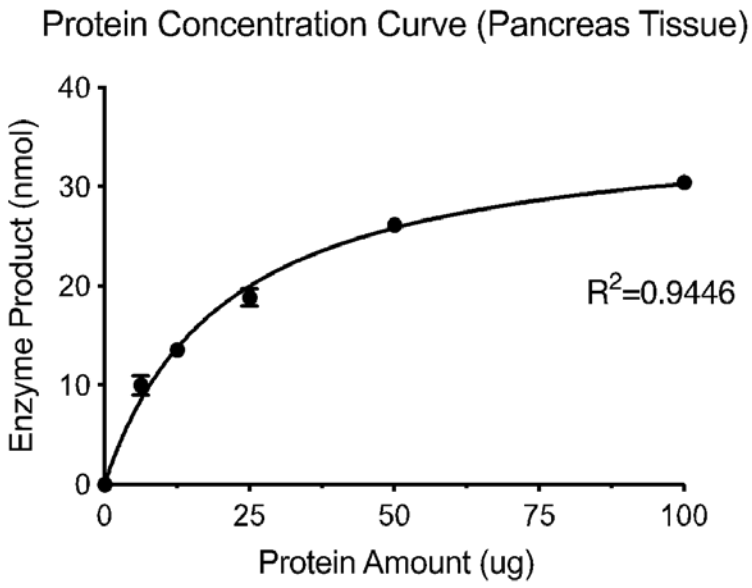

Figure 6. Pancreatic tissue protein concentration curve. MGL Assay normalized with a BCA Protein Assay. R^2=0.9446 which shows a strong and reliable data set. At protein quantities of 50 μg and above, enzyme product begins to plateau, and no more significant product can be made.

A practice MGL assay was conducted using samples from mice on a 60-day experimental setup using a new "standard" diet being studied to compare with the Standard chow diet used as the control. No significant change was expected to be seen between the two diets and can be translated as control pancreatic tissues. Total protein concentration quantities from pancreatic tissues were measured via BCA protein quantification assay and were normalized to 1mg/ml. The results of the procedure were analyzed as seen in **Figure 6** and we attained a viable graph confirming pancreatic tissue quantification.

When looking at the R^2 value of the graph attained, 0.9446, we see a strong successful run as we had a large amount of protein present and our next step would be to formulate an inhibition curve to make further conclusions regarding enzymatic activity. **Figure 6** shows that enzymatic activity plateaus at a protein concentration of roughly 50 μg and no more significant product can be made. Moreover, it also indicates that at roughly 12.5 μg protein concentration we would be able to see differences in enzyme activity when comparing mice pancreas on different diets. Therefore, when formulating the inhibition curve, using a set protein

concentration downshifted to 10 μg shall present comparative values. Overall, these initial experiments allowed for the confirmation that varying degrees of enzymatic activity are quantifiable within pancreatic tissue. Conducting these practice assays have allowed me to see the proper methodology needed to work with the proper amount of pancreatic tissue in order to effectively measure endocannabinoid metabolic enzyme activity within the pancreas.

Conclusion

Before working with the project samples, it was a great recommendation to practice all the techniques that would be performed on pancreatic tissue from other ongoing projects in the lab where pancreatic tissue was not directly being studied. Near March 2020, we would have been ready to work with the actual samples for this project, however, time was cut short due to COVID-19 and we were unable to perform experiments on any of the set methodological samples. With these successful practice runs, as seen in **Figure 6**, we feel confident to run the set methodological samples. Hopefully, in the near future, we are able to apply these techniques and complete this experiment or see the results done by another experimenter.

Our experimental data of future enzymatic assays could show two variations. One would be a curve that reflects the mRNA expression levels as seen in **Figure 3**. If the enzymatic activity matched their respective mRNA expression levels, then we would observe higher 2-AG endocannabinoid levels in the Western diet mice. However, the endocannabinoid levels were the same between the two diets, thus, it would show that some other factors during metabolism are playing a role in the endocannabinoid levels. In addition to MAGL, it is known that ABHD6 and ABDH12 are also enzymes that are able to breakdown 2-AG (Poursharifi 2017). Therefore, although we see an increase in DAGL mRNA expression and a decrease in MAGL expression; the decrease in MAGL expression could be balanced with ABHD6 and ABHD12 activity to show the unchanged 2-AG levels in **Figure 4**.

Another possible result could have been seen enzyme activity that does not reflect the seen mRNA expression levels. This would lead us to conclude that depending on the activity, there were possible post-transcriptional or post-translational modifications (epigenetic

modifications) that could have affected the enzymatic mRNA or resulting protein and have contributed to their activity, giving us the seen endocannabinoid levels.

Studying the epigenetic modifications may shed light on a possible new factor influencing endocannabinoid levels in the pancreas. Even before and after a gene has been transcribed, expression is regulated by many different processes. Factors that may be influencing this role include DNA methylation, post-translation modifications of histones, and small RNA molecules (noncoding RNAs) that influence protein production (Szutorisz 2017). The interaction with all these factors ultimately contributes to gene expression. Studies have shown the implications of epigenetic modifications involved in the endocannabinoid system (Szutorisz 2017). DNA hypermethylation has been observed of the CB1 and FAAH gene promoters under certain diseases leading to variation in endocannabinoid signaling (Szutorisz 2017). This suggests a possibility of hypermethylation of the MAGL promoter in the Western diet resulting in expression levels seen in **Figure 3**, leading to a decrease in MAGL mRNA expression (Szutorisz 2017). In relation to these modifications, another possibility that results in no significant endocannabinoid level changes in between the diets could be from small regulatory RNAs that are degrading the synthetic enzyme mRNA. This test could offer a new viable discovery in the endocannabinoid field where small RNAs are beginning to play a role in endocannabinoid signaling (Szutorisz 2017).

One other possible study would be to look into transcriptional factors between the two diets. In eukaryotes, transcription is controlled by various basal and specific transcription factors. A quantification in transcription factors may lead us to conclusions as to what is causing this increase in mRNA expression in Western diet mice. There may be DAGL transcription enhancers or even MAGL transcription silencers that may be contributing to the relative mRNA

abundance observed. These factors can also be regulated by alterations in nucleosomes and how accessible the transcription binding sites are to the transcription factors recruiting transcription machinery (Szutorisz 2017). This study can possibly help develop medicinal or therapeutic factors that target the transcription factors, whether be silencers, enhancers, etc. that affect endocannabinoid signaling.

Furthermore, mRNA expression does not directly correlate with translation efficiency. Based on stability and epigenetic factors discussed earlier, mRNA's carry a translation efficiency. In correlation with our project, even though DAGL mRNA expression is high compared to MAGL mRNA in Western diet mice, DAGL mRNA may not have high translation rate per mRNA. Similarly, vice versa, MAGL mRNA may have high translation turnover. Ultimately, a combination of these two factors may result in relatively similar 2-AG levels as seen in **Figure 4**.

To further expand upon the study in the mouse pancreas it would be helpful to quantify CB1 receptor density in the pancreas, more specifically in the pancreatic islets, if possible, since that is where insulin production takes place. Even though endocannabinoid levels remained similar between mice on the two diets, an increase in endocannabinoid signaling in the Western diet may come from a potentially larger CB1 density in the pancreas, producing a hyperactive signaling response. Comparing CB1 receptor density between mice tissue on the Standard diet and the Western diet may lead us to further conclusions as to why endocannabinoid signaling is hyperactive in obese mice. Validity from this experiment may suggest further roles of the endocannabinoid system in the development of Type 2 Diabetes and may allow for a creation of therapeutics that may assist insulin regulation and signaling to prevent the onset of diabetes and contribute largely to the medical field.

Potential Limitations

Quantification and potential measurements of the endocannabinoid system substituents were done using the entirety of the pancreatic tissue. When studying insulin regulation, it may be ideal to, instead, quantify the pancreatic islets specifically, however, it would be a very difficult task. A possible avenue of experimentation would be to use more sensitive approaches such as immunohistochemistry (IHC) studies or flow cytometry (FCM) to measure cell sorting and molecular characteristics of specific cells in the pancreatic islets. Subsequently, even though we see no significant change in endocannabinoid levels in the pancreas overall, there may be a change when comparing pancreatic islets between the two diets specifically. This may lead us to further conclusions as to why hyperactive endocannabinoid signaling is produced and how it may relate to the onset of Type 2 Diabetes.

A further limitation to this study and the study conducted in association in the past is that the mice were only on their respective diets for 60-days. 60-days may not be enough time to produce an onset of diabetes or a metabolic disease in general. Therefore, allowing an ample amount of time and monitoring on these diets would be ideal, however, this process may not be ideal in a laboratory condition in terms of efficiency.

Finally, the endocannabinoid system takes part in a large percentage of our body system and it is integrated throughout the endocrine system, gastrointestinal system, and even the central nervous system. Therefore, looking at the endocannabinoid system with a holistic view is necessary to compare when dealing with endocannabinoid signaling. For example, signaling is not only done through 2-AG, but also through other endocannabinoids, like AEA, as well. Evidence has shown that AEA plays a role in pancreatic tissue and signaling through the TRPV1 receptor (DiMarzo 2008). Furthermore, studies show that under certain conditions TRPV1

stimulation inhibits 2-AG biosynthesis (DiMarzo 2008). This may serve as a possible ingredient to unchanged endocannabinoid levels, despite significant higher levels in DAGL mRNA in Western diet mice.

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