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Effect of Alpha-Linolenic Acid on Global Fatty Oxidation in Adipocytes and Skeletal Muscle Cells

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Abstract:

Omega-3 (n-3) and omega-6 (n-6) fatty acids have been known to be essential for normal growth and dermal functions since the 1930s, and multiple benefits of n-3 fatty acids have been recognized over the last 20 years. α-linoleic acid (ALA), an omega-3 fatty acid, is of particular interest because of its suspected role in up-regulating global fatty acid oxidation in adipocytes and skeletal muscle cells. ALA is a substrate for β-oxidation in humans, and also can be elongated to longer-chain polyunsaturated fatty acids (PUFAs) in the endoplasmic reticulum via interactions with desaturase and elongase enzymes. Under long-chain PUFAs deficient conditions, ALA would be converted, but under normal conditions the ALA is competitively inhibited from interacting with Δ6-Desaturase enzyme. N-3 and n-6 fatty acids are metabolized by the same desaturation/elongations pathway; therefore, there exists the potential for competitive inhibition when there are sufficient intracellular concentrations of both families of fatty acids. The logical question that arises is: what happens to the ALA not allowed to participate in the desaturase/elongation pathway? Clearly, it is going to be metabolized, but what mechanisms are involved in this process and are they ALA-specific? The hypothesis is that the mechanisms are not ALA-specific; therefore, the metabolism of surplus ALA will affect the metabolism of other fatty acids. To determine if surplus ALA regulates global fatty oxidation, experiments were ran using the C2C12 cell line of mouse adipocytes. After cells were grown, differentiated, and labeled with ^{3}H-palmitate, 15 different treatments containing various concentrations of ALA, arachidonic acid (ARA), eicosapentaenoic acid (EPA), and docosahexenoic acid (DHA) were added in vitro. The treatments that contained all four n-3 fatty acids mimicked *in vivo* conditions (25μM ARA, 25μM 3:2 EPA:DHA) . Initial experiments show a 141%, 41%, 195%, and 97% increase in fat oxidation in response to progressive increases in ALA from 0 to 10, 25, 50, and 100μM ALA, respectively. These data show that ALA does affect the oxidation of other fatty acids, although, the subsequent experiment did not replicate the initial's results. Therefore, more experiments need to be run to address the lack of fatty acid oxidation dose-responsiveness. If in fact the initial experiment's observations hold true, some hypothesized mechanisms that might cause up-regulated of fatty acid oxidation are increased activity of Carnitine Palmitoyl Transferase I (CPT-I), the ligand-activated transcriptional factors peroxisomal proliferator activated receptor-α (PPARα) and PPARδ, and such downstream effects as increased mitochondrial biogenesis.

Introduction:

Obesity has become an obvious killer in not only America, but throughout the world. A combination of work-related stress, sedentary lifestyle, and poor nutrition has created and allowed the prevalence of obesity to more than double since 1980[1]. Now, 65% of the world's population lives in countries where obesity-related deaths have overtaken non-obesity-related deaths[1]. Luckily, obesity is a preventable condition, and as the prevalence of obesity continues to grow throughout the world, there is increasing interest in a possible solution amongst the scientific community.

Previous Research:

This project evaluates the effect of a specific omega-3 (n-3) polyunsaturated fatty acid (PUFA), but a more general perspective on the history of n-3 PUFA research is necessary to understand the reasoning which led to this study's focus.

Numerous studies have used diet-induced and genetically obese mice to study the effects of n-3 PUFA on mice, effects such as: weight loss, fat reduction, and protective benefits against the accumulation of body fat[2]. The diets among certain studies varied. Usually, the mice were categorized into two groups, two high-fat diets with the second group being supplemented with fish oil or EPA/DHA, a high-fat supplemented diet and low-fat/control diets, or a multiple high-fat diets supplemented with different absolute amounts of EPA/DHA or fish oil[3].

Multiple studies have supported the claim that long chain n-3 PUFA have protective effects regarding body fat accumulation. Hainault *et al.* found that, although body weight did not change, Wistar rats that consumed a lard-fish oil diet (33% lard, 20% casein, 30% starch, 2% corn oil, 15% fish oil) had 20% less inguinal adipose tissue and 30% retroperitoneal and epididymal adipose tissue after the 20-day study[4]. Parrish *et al.* found through a high-fat (20% triglycerides from fish oil or 20% from lard oil) diet study, his Wistar rats in the fish oil group did not differ in body weight from the lard oil group, but Parrish saw a significant reduction in perirenal and epididymal fat from the lard oil group[2]. Several more studies delved further into the correlation between n-3 PUFA supplementation and fat reduction, specifically discovering a dose-dependent relationship[5] and suggestion that DHA is primarily responsible for encouraging reduction of cellularity and attenuation of visceral fat accumulation[6].

The accumulating evidence for the beneficial effects of long chain n-3 PUFA is exciting. Though, for the sake of fair argument, one 2006 study did offer contradicting evidence. Todoric *et al.* examined body weight, metabolic changes, and genes regulating the inflammatory response in C57BL/KsJ-lepr^{db}/lepr^{db} diabetes mice on high-fat diets[7]. He found that those mice on a high-fat diet (30% of energy) which were given a n-3 PUFA-rich supplement actually had significant weight gain compared with the mice on a n-6 PUFA-rich diet[7]. Although body fat was not a parameter measured in the Todoric *et al.* study, the findings do lend to the speculation that long chain n-3 PUFA have different metabolic impacts on mice with diabetes[3]. Contrastingly, the study also provides support for n-3 PUFA interference with white adipose tissue proinflammatory response of mice on obese mice[7].

α-linolenic acid:

α-linolenic acid (18:3n-3, ALA) is an essential n-3 PUFA in the human diet and is the principal fatty acid in the western diet[8]. ALA is converted to longer-chain PUFA in the endoplasmic reticulum of the liver[10] of human adults, and is of particular interest since the conversion pathway yields the PUFA eicosapentaenoic acid (EPA) (20:5n-3) and docosapentaenoic acid (DHA) (22:6n-3). Figure 1 provides a general pathway for ALA conversion to longer n-3 PUFA. Studies have not yet shown if the Δ6-Desaturase enzymes that participate in the conversion pathway are the same enzyme or two distinct ones. Also, both n-3 and n-6 PUFA are metabolized by the same desaturation/elongation pathway, so, therefore, there exists the potential for competitive competition between n-3 and n-6 PUFA[8]. In fact, Δ6-Desaturase has a greater affinity for ALA although linoleic acid is at greater cellular concentration than ALA, causing greater conversion of the n-6 PUFA. The effect of increased consumption of ALA is of interest because of the supposed increase of n-3 PUFA conversion if ALA intracellular stores were to be increased. Also, the effect of sufficient amounts of intracellular EPA/DHA, specifically DHA since it has been found to be the primary parameter affecting visceral fat accumulation[6], is of interest since competition for Δ6-Desaturase within the pathway would lead to feedback inhibition. In fact, the conclusion of a 1994 ^{13}C- isotope

incorporation study stated that the conversion of ALA, which entered the desaturase/elongase pathway, to DHA is 4%[9]; while a 2002 study of young men found no detectable radioactive DHA up to 21 days[10]. Tang *et al.* found that n-3 and n-6 PUFA suppressed

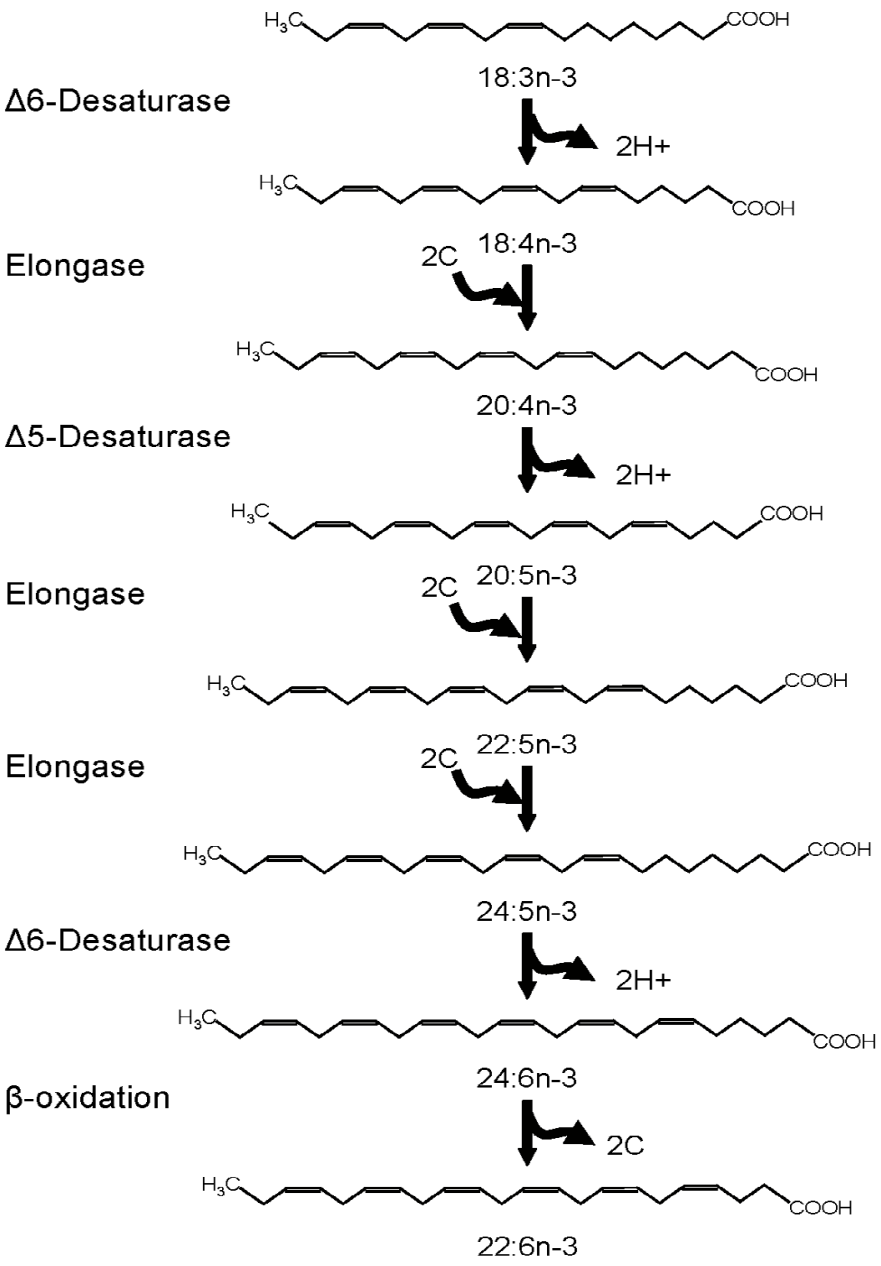

Figure 1: General pathway for ALA conversion through multiple desaturase and elongase steps (based on reference 8). The initial reaction is the rate-limiting step. 24:6n-3 is translocation from the ER to the peroxisome where the acyl chain is shortened. 22:6n-3 (DHA) is translocated back to the ER[8].

hepatic expression of rodent Δ6-Desaturase by inhibiting its gene transcription[11]. Specifically, Δ6-Desaturase contains a response element for the ligand-activated transcription factor peroxisomal proliferator activated receptor-α (PPARα) in its promoter region, and DHA was found to bind to PPARα thus promoting the previously mentioned inhibition of Δ6-Desaturase gene transcription[8, 11]. The studies lead to the conclusion that feedback inhibition of the desaturase/elongase pathway by DHA would result in even lower conversion of ALA to EPA and DHA.

With this knowledge about the feedback inhibition of ALA conversion pathway, a logical question would be: what happens to the ALA if it does not participate in the conversion to longer chain n-3 PUFA? We suspect that the excess ALA plays a role in up-regulation of global fatty acid oxidation in adipocytes and skeletal muscle cells. The excess ALA is most likely metabolized, but since an ALA-specific oxidation mechanism has not yet been discovered, nor is one postulated to exist, the increase in ALA oxidation would increase other fatty acid metabolisms as well.

Materials/Methods:

To determine if surplus ALA regulates global fatty oxidation, experiments were ran using the C2C12 cell line of mouse adipocytes.

Initial Preparation:

Initial C2C12 mouse adipose tissue line was withdrawn from liquid nitrogen and thawed. The thawed C2C12 cells were then introduced into a 75cm^2 canted neck flask (T75) from Fisher Scientific™ . The thawed cells were fed with phenol red Dulbecco's Modified Eagle Medium (DMEM) high glucose1X (GIBCO®). Penicillin-streptomycin (pen-strep), an antibiotic, and Fetal Bovine Serum (FBS), a serum-supplement with low level of antibodies and high level of growth factors, were added at 1% and 10% solution composition, respectively, in order to diminish risk of contamination and encourage cell growth. The cells were always kept in a 37^oC incubator when not examined or re-fed. The DMEM/10%FBS/1%pen-strep (growth medium) was changed everyday until the cells became 80-90% confluent. When cells were 80-90% confluent, they were washed with Hank's Balanced Salt Solution (HBSS) 3 times, then washed with trypsin-EDTA 1X (Mediatech™), spun down in 50mL falcon tube to remove supernatant, and then the pellet of cells were re-suspended in 210mL of the growth medium. Then, the dilution was split amongst 8x24 well-plates, with 1mL of solution into each well for a total of 192 wells. The growth medium was changed everyday. When the cells in the well-plates became 80-90% confluent, a new DMEM/2%Horse Serum/1%pen-strep (differentiation medium) was mixed to promote cell differentiation. After 3 days of differentiation medium supplementation, a new medium serum-free mixture was created containing DMEM/0.2%Horse Serum/1%pen-strep. The cells were allowed to incubate for 24 hours in the serum-free mixture before they were ready for the fatty acids treatments.

Treatments:

Fifteen different treatments were used. The main fatty acids used were arachidonic acid (ARA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DHA) and α-linlenic acid (ALA). There were 3 main groups, each with the same 5 ALA concentrations, yielding a total of 15 treatments. The 3 main treatment groups were: the control (solvent), 25μM ARA, and 25μM 3:2 EPA:DHA. Each group had the same 5 ALA concentrations mixed into the solution: 0, 10, 25,

50, and 100µM ALA. The solvent for the treatments was DMEM + Bovine Serum Albumin, which acts as a transport protein for the fatty acids into the cells.

30X ALA, ARA, and EPA:DHA concentrations were made up from the ethanol dissolved pure fatty acids. These 30X concentrations were then diluted into the final concentrations by the solvent. The solvent composed of 495mg of BSA for every 15mL of DMEM needed. To have sufficient fatty acid treatments, 15mL of each of the 15 treatments were made up and 1mL of the appropriate treatment was added to the 12 corresponding wells. The treatments were incubated at $37^{o}C/5\%CO_2$ for 12-16 hours.

Fatty Acid Oxidation Protocol:

Reaction Mixture: There The 12 wells per treatment were split into 6 positive, tritium(5µCi)-labeled protein wells and 6 negative, formalin(10% Phosphate Buffered Solution)-labeled wells. The 6 negative wells were labeled with formalin in order to kill the cells and ensure there would be no activity except background counts recorded. This provides high count contrast in relation to the tritium-labeled wells and ensures the recorded radioactive was not just background radioactivity. The reaction mixture added to each well composed of 20mL HBSS, 10 mg BSA, 200µl unlabeled palmitate, and 531µl tritium for the positive controls. For the negative controls, 531µl of formalin was added, replacing the tritium.

Addition/Incubation: Once the reaction mixture was made, each well received 200 µl of the appropriate mixture. Any wells that exhibited contamination from the previous ALA treatments were skipped. After the addition of the appropriate mixtures, the well plates were incubated at $37^{o}C/5\%$ CO_2 for 2 hours.

Protocol Preparation: Chromatography columns with resin solution were prepared the night before the protocol was run. A total of 180 columns were sorted onto racks, and 2. mL of resin solution was added to each column. The resin solution composed of 20 g of Dowex ® 1X2, 200-400 mesh, ion-exchange resin (Acros Organics®) for every 55mL of MilliPore® filtered water. A large beaker was used to contain sufficient resin solution for the 180 columns, and the solution was constantly stirred via magnet stirring rod until all columns had 2.5mL resin solution. They were allowed to sit overnight. Also, the night before and/or during the 2 hour incubation, 2 sets of 180 2mL vials were labeled 1-180 and ordered onto racks for use later in the protocol. Also, 200µl of trichloroacetic acid (TCA) was added to each tube during the 2 hour incubation period. The TCA should be 10% wt/vol.

Protocol Procedure: After the 2 hour incubation period, all 8 well-plates were taken out of the incubator. The mixture from each well was removed and placed into itself appropriately labeled 2mL vial. When the entire well is finished, 100µl of Phosphate Buffered Saline (PBS) was added to each well. After 1 minute, the PBS was removed and added to its appropriately labeled vial. After the vials sat for 2 minutes, they were centrifuged at 8,500g for 5 minutes. At this time, the twist-off caps at the bottom of the chromatography columns were broken, allowing the water to sift through the now-settled resin. After the 5 minute centrifuge wait, the supernatant was removed immediately and placed into the 2^{nd} set of appropriately labeled 2mL vials. A 70µl addition of 6N NaOH proceeded in order to counteract the TCA. The supernatant/NaOH mixture was then transferred to its appropriately labeled chromatography column. Each column then received 1mL of MilliPore® filtered water to facilitate the wash of the supernatant/NaOH mixture through the resin. The filtrate was collected in an empty scintillation vial and left overnight. The well-plates now only contained the cell layer, so 500µl of MilliPore® filtered water was added to each well. The well-plate was then taped shut and put into a $-80^{o}C$ freezer

until ready for the protein correction protocol. The next day, 10mL of Bio-Safe II™ scintillation fluid was added to each scintillation vial. The vials were then put into a liquid scintillation counter which recorded the counts per minute (CPMs) at a 1 minute rate. The vials were then taken out and kept behind a radioactive-material protective hood.

Protein Correction:

Once the CPMs for each well were recorded, a protein correction was run either using the Commassie Protein Assay Kit (Thermo Scientific ®) or the BCA protein assay reagent kit (Thermo Scientfic ®). A spectrophotometer assisted by a spectral count program was used to interpret the data into numerical form. Given this information, the corrected CPM/μg of protein erased the cell population/well discrepancy from well-to-well.

Results:

Data/Interpretation:

The initial experiment data, for the 3:2 25μM EPA:DHA treatments, show a 141%, 41%, 195%, and 97% increase in fat oxidation in response to progressive increases in ALA from 0 to 10, 25, 50, and 100μM ALA concentrations, respectively (Figure 2). Also, the 25μM ARA treatments show an initial decrease of 4.1%, but then a rebound of 31%, 266% before another loss in oxidation of 32% in response to progressive increases in ALA from 0 to 10, 25, 50, and 100μM ALA, respectively (Figure 3). The initial data for both EPA:DHA and ARA treatments show increases in fat oxidation for palmitate (16:0). There was an observed 33% and 82% decrease in fat oxidation from 50 to 100μM ALA concentrations within the EPA:DHA and ARA treatments, respectively, suggesting a possible threshold for increasing oxidation at 50μM ALA for both treatments.

For the subsequent experiment, the EPA:DHA treatments showed decreases of 47%, 30%, 36%, and 34% in fat oxidation from 0 to 10, 25, 50, and 100μM ALA concentrations, respectively. The ARA treatments showed initial increases of 54%, 27%, and then decreases of 1.6% and 29%, respectively.

The initial experiment did suggest a ALA-stimulated increase in palmitate oxidation, but only one subsequent experiment was ran due to time constraints and its data did not replicate the initial experiment's. Due to lack of consistent data, this study's interpretation of whether ALA does up-regulate fatty acid oxidation will be left inconclusive until more repeat experiments are run.

Sources of Error:

Similar to any scientific experiment, there are possible sources of error that should be enumerated in order to maintain scientific integrity.

Aside from any inherent human error, certain initial and subsequent ARA and EPA:DHA treatments did not have the full 6 positive and 6 negative controls as listed in the *Fatty Acid Oxidation Protocol* above. In the ARA initial treatments, 0 and 25μM ALA concentrations had 4 and 5 positive controls, respectively. In the EPA:DHA initial treatments, 25μM ALA concentration had 5 positive controls. In the subsequent ARA experiment, 25μM ALA concentration had 5 positive controls. In the subsequent EPA:DHA experiment, 0μM ALA

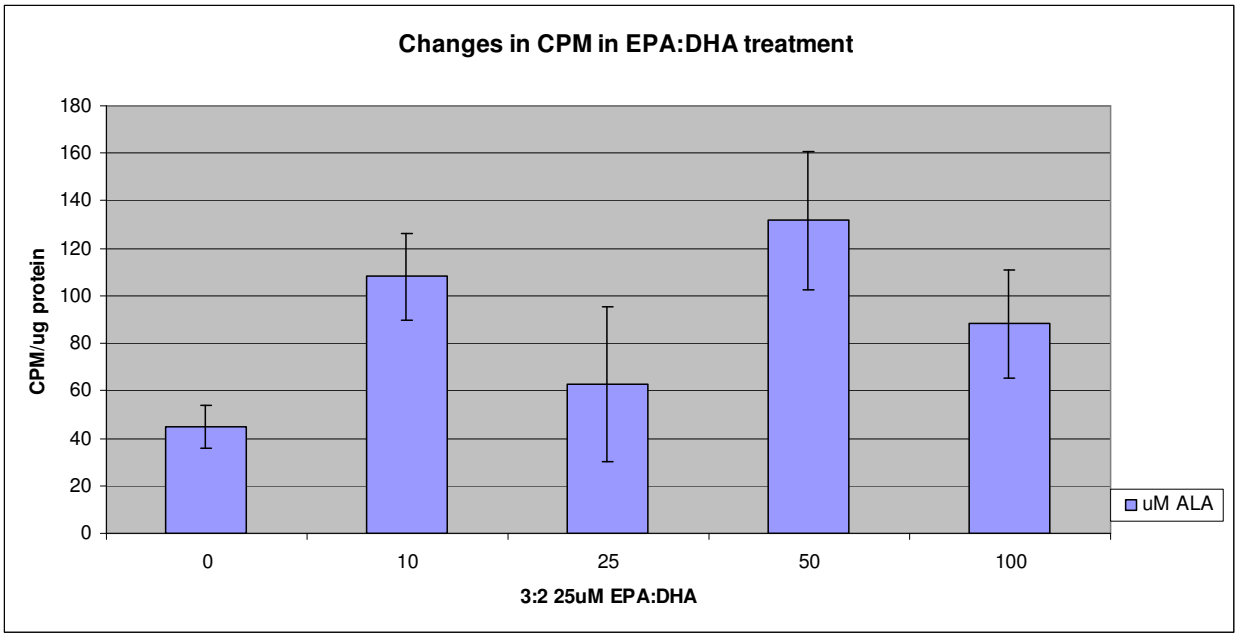

Figure 2: Initial experiment changes in palmitate oxidation at various concentrations of ALA within the EPA:DHA treatments. The data suggests ALA-stimulated increase in fat oxidation and a possible threshold for oxidation around 50uM ALA. Error bars represent the standard error of the mean.

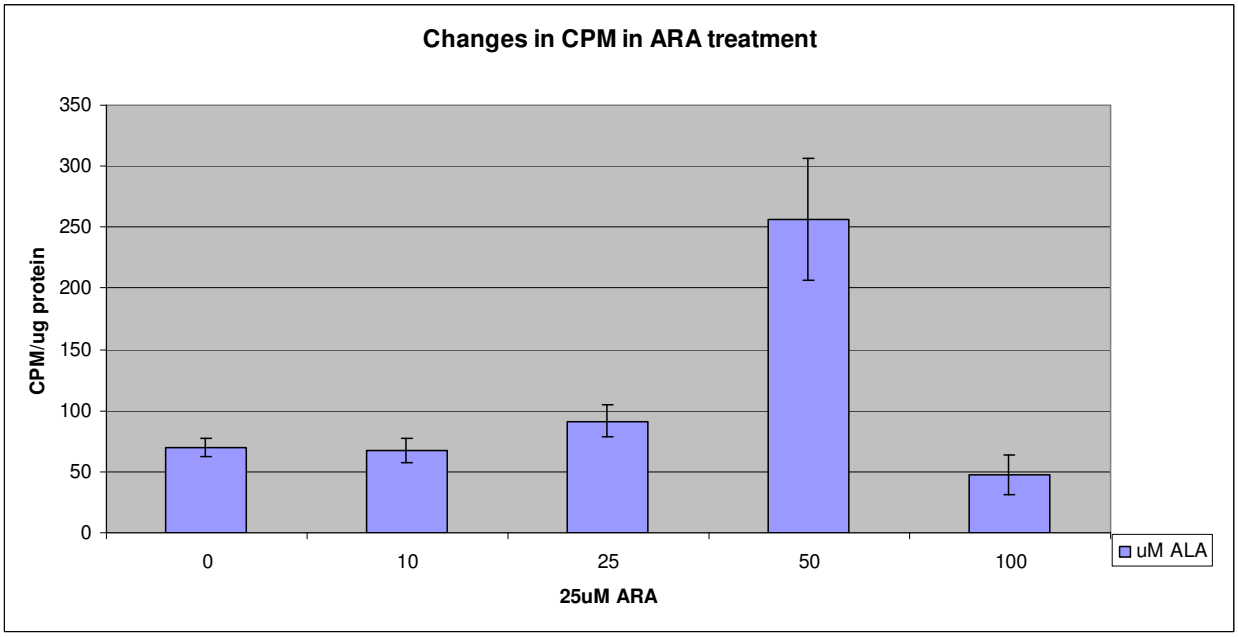

Figure 3: Initial experiment changes in palmitate oxidation at various concentrations of ALA within the ARA treatments. The data also suggests ALA-stimulated increase in fat oxidation and a possible threshold for oxidation around 50uM ALA. Error bars represent the standard error of the mean.

concentration had 5 positive controls. One or two fewer positive, tritium-labeled controls increases the susceptibility of the mean to outliers in the data, therefore, leading to sample data that are not representative of the proposed cell population. A solution would be to run more repeat experiments in the future to obtain more sample data, therefore, making the complete sample data more representative of the proposed cell population.

Another possible source of error stems from the time dedicated to finishing the fatty acid oxidation protocol. With only one researcher working through the protocol, each plate took about 45 minutes to complete meaning that the 8th plate could have sat at room temperature for 6 hours before beginning the protocol after the 2 hour incubation period. This extra time, even though at room temperature, could have affected the recorded CPM of the latter treatment plates. A solution would be to have more than one researcher completing the protocol once started and/or investigating the effects of refrigerating the treatment plates until they are needed after the 2 hour incubation period.

Future/Proposed Experiments:

Multiple repeat experiments will have to be run to obtain consistent data before any of the following hypotheses will be tested.

Mechanisms of Action:

If the repeat experiment suggest ALA dose-dependent up-regulation of fatty acid oxidation response, the next step would be to understand exactly how these increases in fatty acid oxidation are caused.

Carnitine palmitoyltransferase I (CPT1) is one of the cycle carnitine enzymes that transport long chain fatty acids from the cytoplasm into the mitochondria[12] for incorporation into the β-oxidation pathway. Hypothesized mechanisms to investigate are the up-regulation of CPT1, and more specifically CPT1B, the muscle-type isoform of CPT1[12], activity.

Also, there is significant interest in the role of PPARα and PPARδ, PPAR isoforms, in the regulation of fatty acid oxidation. Studies have found that the PPARs modulated enzymes involved in lipid metabolism, especially in metabolically active tissues such as sketetal and adipose, thus suggesting that they may play a key role in energy metabolism and affect weight gain[8, 13].

Future studies will be run that will investigate these proposed mechanisms of action of fatty acid oxidation regulation. Also, repeat fatty acid oxidation protocol experiments will be ran in order to obtain more sample data, which will better illustrate the observed trends of the 15 treatments.

References:

1. World Health Organization. (2011, March). *Obesity and overweight*. Retrieved from http://www.who.int/mediacentre/factsheets/fs311/en/
2. Parrish C. *et al*. Dietary fish oils limit adipose tissue hypertrophy in rats. *Metabolism* 1990, *39*, 217-219.
3. Buckley, J.D. & Howe, P.R.C. Long-Chain Omega-3 Polyunsaturated Fatty Acids May Be beneficial for Reducing Obesity—A Review. *Nutrients* 2010, *2*, 1212-1230.
4. Hainault, I. *et al.* Fish oil in a high lard diets prevents obesity, hyperlipidemia, and adipocyte insulin resistance in rats. *Ann. N.Y. Acad. Sci.* 1993, *683*, 98-101.
5. Belzung, F.; Raclot, T. & Groscolas, R. Fish oil n-3 fatty acids selectively limit the hypertrophy of abdominal fat depots in growing rats fed high-fats diets. *Am. J. Physiol.* 1993, *264*, R1111-R1118.
6. Ruzickova, J. *et al.* Omega-3 PUFA of marine origin limit diet-induced obesity in mice by reducing cellularity of adipose tissue. *Lipids* 2004, *39*, 1177-1185.
7. Todoric, J. *et al.* Adipose tissure inflammation induced by high-fat diet in obese diabetic mice is prevented by n-3 polyunsaturated fatty acids. *Diabetologia* 2006, *49*, 2109-2119.
8. Burdge, G.C. & Calder, P.C. Conversion of α-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. *Nutr. Dev* 2005, *45*, 581-597.
9. Emken, E.A. *et al.* Dietary linoleic acid influences desaturation and acylation of deuterium-labeled linoleic and linolenic acids in young adult males. *Biochim Biophys Acta* 1994, *1213*, 277-288.
10. Burdge, G.C. *et al.* Eicosapentaenoic and docosapentaenoic acids are the principal products of α-linlenic acid metabolism in young men. *Br J Nutr* 2002, *88*, 335-363.
11. Tang, C. *et al.* Regulation of human Δ-6 desaturase gene transcription: identification of a functional direct repeat-1 element. *J Lipid Res* 2003, *44*, 686-695.
12. Ogawa, E., *et al.* Expression analysis of two mutations in caritine palmitoyltransferase IA deficiency. *J Hum Genet* 2002, *47*, 342-347.
13. Harrington, W.W., *et al.* The Effect of PPARα, PPARβ, PPARγ, and PPARpan Agonists on Body Weight, Body Mass, and Serum Lipid Profiles in Diet-Induced Obese AKR/J Mice. *PPAR Res* 2007, 97125.