

**EX-VIVO CHARACTERIZATION OF THE LIPOLYTIC RESPONSE OF
MOUSE ADIPOSE TISSUE TO ENDOCRINE STIMULI**

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ABSTRACT

The state of obesity can change the metabolic state of adipocytes, causing disorders in their lipolytic response to endocrine stimuli. Fasting has been proven to improve insulin sensitivity thereby increasing the cellular uptake of glucose which affects lipolysis. To better characterize the endocrine function of visceral white adipose tissue (WAT), an ex-vivo model using tissue explants from fasted and non-fasted DBA/2J mice was tested. Isoprenalolol and forskolin, β -adrenergic (β A) and adenylyl cyclase (AC) receptor stimulators, respectively, were used as endocrine stimulators. Lipolysis was measured by the concentrations of non-esterified fatty acid (NEFA) and glycerol in the media. Fasted mice showed a more than 10-fold increase in NEFA secretion than non-fasted mice when treated with isoprenalolol. Further, direct stimulation of β A increased lipolysis significantly more than AC stimulation in mouse WAT. Together, these findings indicate that endocrine stimuli play a major role in the lipolytic response of visceral WAT.

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BACKGROUND

Obesity

As defined by the World Health Organization, being obese or overweight is the state of having accumulated excessive fat (World Health Organization, 2013). This surplus of body fat triggers a myriad of major health risks that shorten the lifespan of afflicted individuals, and increase the incidence of major diseases such as coronary heart disease, Type 2 diabetes, hypertension, and osteoarthritis. Although obesity can be preventable, in 2008 the number of adults over the age of 20 diagnosed as obese was 200 million men, and 300 million women. Sixty five percent of the world's populations live in countries where obesity and overweight kill more people than being underweight (Obesity and overweight, 2013).

Obesity is one of the two major lifestyles that affect the metabolic function of organisms; the other is fasting. Becoming a North American epidemic in recent years, obesity, can cause detrimental effects on metabolic functions. Fasting is the abstention from consuming food, and similar to obesity, alters the function of adipose tissue. Obesity or fasting cause extreme metabolic states linked to disorders in the lipolytic response of adipose tissue to proper stimuli. Understandably, these alterations take a major toll on the immune system, metabolism, and hormone balance, which are all connected through many biochemical pathways.

Inflammation

The word inflammation derives from the Latin word *inflammare* meaning “to ignite.” There are two types of inflammation: classic inflammation and silent inflammation. The body’s first line of defense, the innate immune system, uses inflammation as one of the first responses to destructive stimuli. In classic inflammation, the innate immune system can cause pain observed as a red, achy, irritation on the surface of the skin. The classic inflammatory response can also be defined as a non-specific immune response that occurs in reaction to broad number of harmful stimuli (Ferrero-Miliani et al., 2007). The latter is also called chronic inflammation and has no pain association. Because of this characteristic of silent/chronic inflammation, medically, nothing is often done to treat the tissue damage, so the damage can continue for years until irreparable damage has transpired to organs (Sears and Ricordi, 2011).

Eicosanoids are the primary drivers of the body’s inflammatory response. This umbrella term is used to describe both pro-inflammatory agents and anti-inflammatory agents (Ferrero-Miliani et al., 2007). Anti-inflammatory eicosanoids are derived from omega-3 fatty acids, such as eicosapentaenoic acid. Pro-inflammatory eicosanoids are produced from omega-6 fatty acids such as arachidonic acid (Smith, 1989).

Arachidonic acid is a polyunsaturated fatty acid found abundantly within phospholipids of cell membranes. Specific stimuli act on the cell membrane to allow phospholipases to cleave phospholipids, and arachidonic acid is released (**Figure-1**). This acid is a proponent of inflammation, and in many cases cellular inflammation begins with that step. Oxygenation metabolizes arachidonic acid into products such as prostaglandin

PGD₂ or PGE₂ that drive an immune response (Davies et al., 1984). Healthy organisms have a balance of these inflammatory agents, but when there is an imbalance in the ratio of arachidonic to eicosapentainoic acids in the blood, silent inflammation initiates various organs, as the pro-inflammatory agents are not being balanced by enough anti-inflammatory agents (Smith, 1989; Sears and Ricordi, 2011).

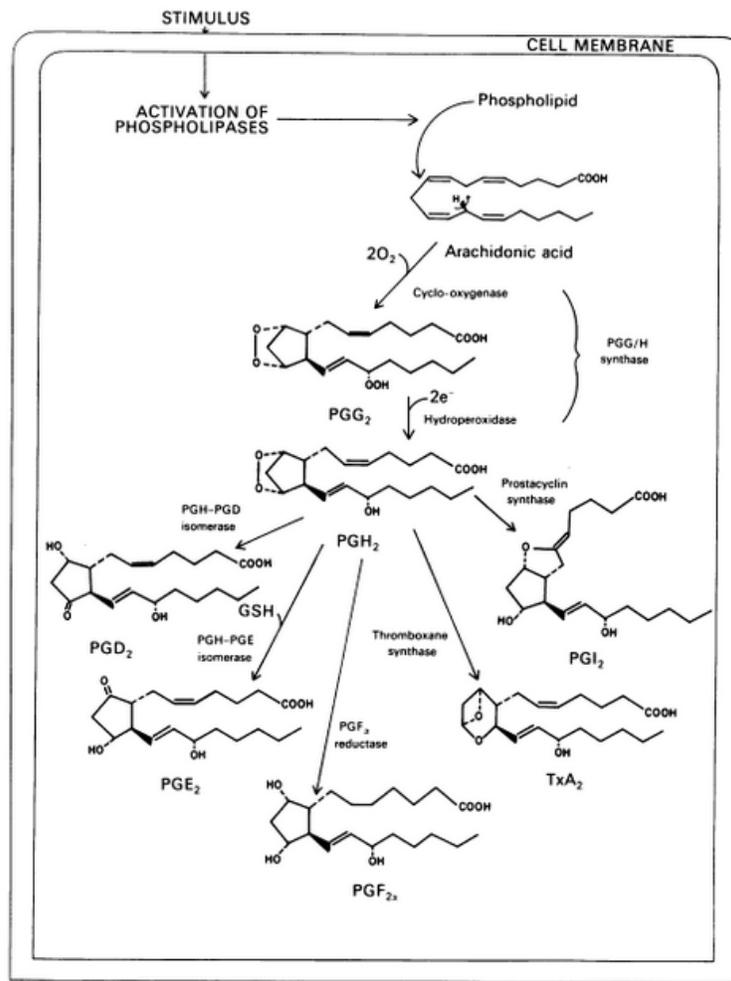


Figure-1: Biosynthetic Pathway of Prostaglandin Formation. Shown is the pathway involved in the oxygenation of arachidonic acid through cellular stimulus of phospholipids to produce prostaglandins. PG, prostaglandins; Tx, thromboxane. (Sears and Ricordi, 2011)

Obesity and Inflammation

The levels of Omega-6 fatty acids and omega-3 fatty acids within organisms are wholly controlled by the diet. In recent years, the prevalence of omega-6 fatty acids has increased in processed foods through linoleic acid, common in cheap vegetable oils. However, the consumption of flaxseeds, walnuts, or salmon, which are all rich in omega-3 fatty acids, has diminished over the past decade. As previously mentioned, arachidonic acid is an omega-6 fatty acid that promotes pro-inflammatory agents in the body, while omega-3 fatty acids do the opposite. The most prevalent, least costly foods are often ridden with pro-inflammatory fatty acids. Becoming obese usually requires the consumption of large quantities of these foods accompanied by an inactive lifestyle. For example, osteoarthritis, the degradation of the joints from *classic* inflammation, is very prevalent in the obese population and is associated with a great deal of pain. Another example is type 2 diabetes, also prevalent in the obese population, which is associated with *silent* inflammation that causes insulin resistance. Thus, the state of being obese or overweight is intimately connected with the regulation of both forms of inflammation by the innate immune system (Sears and Ricordi, 2011).

Energy Storage and Metabolism

Organisms use sugars and fats as energy sources to function properly. Sugars, more formally known as carbohydrates, are the primary source of energy in animals. Fats, on the other hand, are lipids like triglycerides that play a supporting role as an energy source. While carbohydrates are stored in the form of glycogen in muscle and in the liver, fats are found in

lipid deposits in the form of triacylglycerol. Although disorders can result from abnormal carbohydrate storage, more pressing problems are associated with fat storage and metabolism.

Review of Adipocytes

Adipocytes are fat cells that contain all of the regular organelles found in eukaryotic cells, such as a nucleus, mitochondria, and ribosomes. The hallmark feature of adipocytes is their large intracellular lipid droplets that store triglycerols (**Figure-2**). With the help of lipases, they can be broken down and secreted for cellular consumption (Alberts et al., 2008).

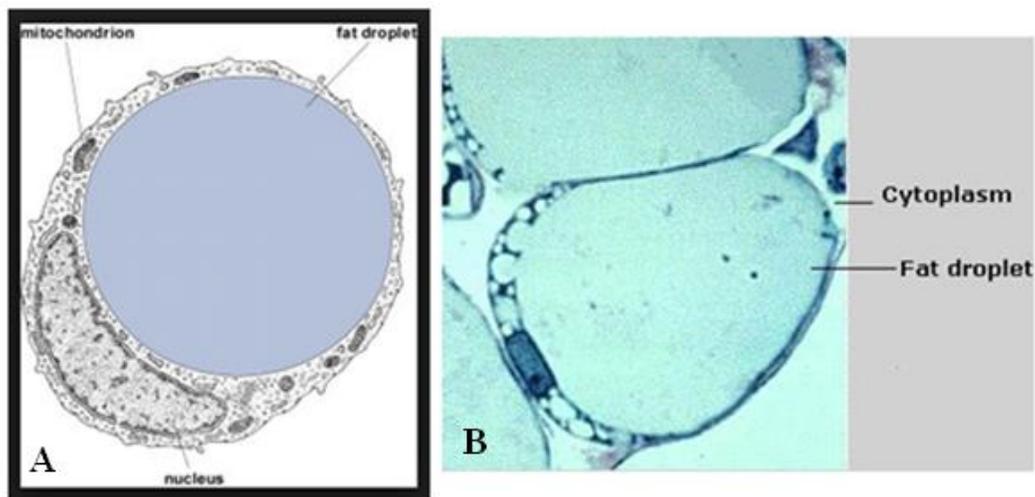


Figure-2: Animated and Microscopic Images of Adipocyte Physiology. **A**, the animated representation of a white adipocyte physiology showing mitochondria, lipid droplet, and nucleus (Lentz and Saunders, 1971); **B**, microscopic representation of a white adipocyte showing the large fat droplet and cytoplasm holding the organelles. (“Structure of Adipose,” tutorvista.com)

Adipocytes play an essential role in energy metabolism. They can be found in subcutaneous (below the skin) or visceral (between organs) depots in the body (Henry et

al., 2011). Adipose tissue is composed of adipocytes. This tissue was originally thought to be inert, with only lipid metabolism and storage as its function; however adipose tissue has recently been identified as also having endocrine functions. Thus its purpose is now grouped into two more main categories with overlapping mechanisms: glucose metabolism and endocrine functions (Ahmadian et al., 2009; Henry et al., 2011).

Obesity Effects on Energy Storage and Metabolism

The size of adipocytes greatly affects their metabolic functions; there is a positive correlation with endocrine dysfunction (Henry et al., 2011). Insulin is produced by the pancreas and acts on cells to increase glucose transport into cells using insulin receptors for energy usage. Adiponectin is an adipocyte-derived hormone that regulates glucose and lipid metabolism through AMP-activated protein kinase by sensitizing cells to insulin which improves energy metabolism. Adiponectin and insulin work together to regulate energy homeostasis (Yamauchi and Kamon, 2001; Qiao et al., 2011).

When excess triglycerides are present, their storage becomes improper within lipid droplets, and negatively affects the function of adipose tissue. One primary function affected by this excess of fat is the production of adiponectin, a hormone that inhibits triglycerol breakdown and induces insulin sensitivity (Qiao et al., 2011). Insulin resistance occurs when cells do not respond properly to insulin stimuli, leaving increased amounts of glucose in the blood and weakened cells with not enough glucose (Health Effects, 2012). The larger sized adipocytes help lead to insulin resistance because the lack of adiponectin production. The super-storage of fat causes a homeostatic hormone imbalance. Therefore, obesity and

excessive fat storage causes insulin resistance, which is the foundation of many diseases such as cardiovascular diseases, type 2 diabetes, and cancer (Henry et al., 2011).

Fasting Effects on Energy Storage and Metabolism

Fasting is the abstention from food or drink which lessens energy intake into the body. Fasting has effects on other cellular mechanisms, such as impaired glucose tolerance, also known as starvation diabetes. Fasting leads to an increase in insulin binding thus effecting insulin cascades, especially glucose entrance into the cell for energy metabolism. Fasting, at first glance, would seem to have a diminishing effect on insulin binding to its receptors. However, observed maximal adipocyte glucose metabolism takes place only when a small portion of the cells' insulin receptors are active (Olefsky, 1976). In other words, fasting stimulates too many of the cells' insulin receptors thereby causing an influx of glucose to the cell and maximal adipocyte glucose metabolism is not observed.

Fasting leads to cellular glucose intolerance, but it also drives gluconeogenesis, which is the metabolic formation of glucose from fats and proteins rather than from carbohydrates. This takes place primarily in the liver through the citric acid cycle of mitochondria, and the reversal of the glycolysis process which would break down glucose into pyruvate yielding free energy (Martin et al., 1981).

Fasting and Lipolysis

Triglycerol breakdown and release from adipocytes, known as lipolysis, also occurs during fasting, as gluconeogenesis requires fatty acids to proceed. Lipases, activated by the

hormone insulin in adipocytes, are the enzymes formed in adipose cells that help store and break down triglycerols into nonesterified fatty acids (NEFA) that can be secreted for consumption by other cells. These non-esterified fatty acids are free single chain fatty acids that, when reacted with the alcohol glycerol (also known as glycerol-3-phosphate), form the esters, triglycerides.

Insulin normally binds with the insulin receptor to initiate signal transduction events that up-regulate the glucose receptor/transporter (GLUT) on the cell surface. GLUT binds serum glucose and brings it into the cell. After glucose has entered the adipocyte, it is broken down into glycerol used to form triglycerides (**Figure-3**). As previously mentioned lipases are hormone-sensitive enzymes that cleave fatty acids from the glycerol backbone of triglyceride molecules. Lipases, and thus lipolysis, are activated in times of a low energy state, to provide NEFA that are stored in adipocytes for use by other tissues such as muscle. In mammals, insulin is the primary hormone that signals the “fed” state, and it is at high levels in the blood when energy is abundant. Insulin promotes the uptake of glucose by cells and inhibits the breakdown of triglycerides. During fasting, insulin levels fall and increased lipolysis is observed. Other hormones such as glucagon and catecholamines also increase during fasting and stimulate lipolysis.

Within the adipocyte, lipolysis is stimulated by adenylate cyclase activation and an increase in the cellular messenger cyclic-AMP (cAMP). Lipolysis is therefore regulated by the balance of pro- and anti-lipolytic hormones present in the bloodstream, and the level of cAMP within the adipocyte. The primary outcome of lipolysis during fasting is to provide NEFA for use by other tissues. However, fasting also increases NEFA locally within adipose tissue, which can alter adipocyte metabolism as well as inflammation (Alberts et al., 2008;

Martin et al., 1981). Therefore in the processes of energy storage and energy metabolism during fasting triggers protein cascades that influence fat storage and adipocytes' hormonal responses. That is why fasting is often used in nutritional studies of adipose tissue, as it jumpstarts the metabolic processes.

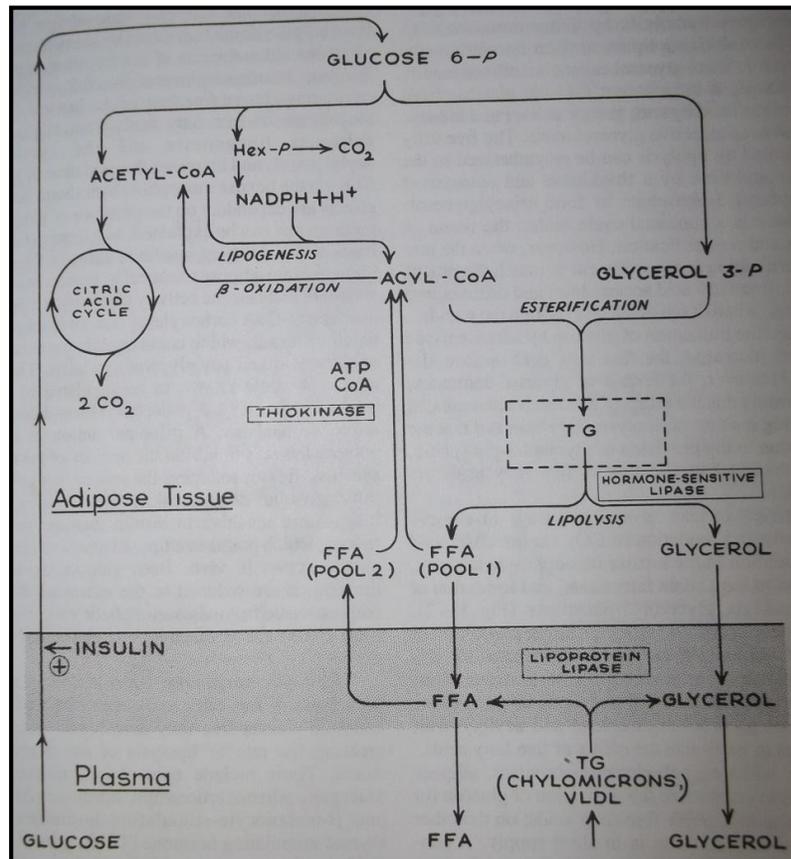


Figure-3. Adipose Tissue Lipogenesis and Insulin Induction of Lipolysis. Shown are two major energy pathways that take place within adipose cells and in the bloodstream that are activated by insulin. Important abbreviations used: TG, Triglycerides; FFA, free fatty acids; VLDL, Very Low Density Lipoprotein (Martin et al., 1981).

Catecholamines

Catecholamines are amines that function as hormones in the body. Epinephrine is one of the most abundant catecholamines in the human body, and it is commonly known as adrenaline. Catecholamines operate through adrenergic receptors (both α and β cell receptors) throughout the body to promote a host of metabolic changes including, in adipose tissue, triggering lipolysis. Isoprenalol, also known as isoprenaline, is a manmade drug that is commonly used in the treatment of slow heart rate. Isoprenalol stimulates lipolysis by first stimulating the β -Adrenergic receptors (β -AR) on the surface of adipocytes. By acting through the same receptors as adrenaline, isoprenalol is considered a catecholamine agonist and thus in adipose tissue it activates lipolysis just as adrenaline would (Allen et al., 1986).

Forskolin is not a catecholamine. Instead it functions by directly stimulating adenylyl cyclase to produce cAMP, and begins the lipolysis stimulation mechanism. Both forskolin and isoprenalol have been used in previous studies to stimulate adenylyl cyclase. As seen in the animation **Figure-4A**, when the β -AR receptor is stimulated, the Gs protein (Gs) of the receptor activates adenylyl cyclase (AC) and begins the cyclic AMP (cAMP)-dependent pathway by producing cAMP. This mechanism stimulates lipolysis because cAMP then activates protein kinase A (PKA) which, through direct phosphorylation of hormone sensitive lipase (HSL), lipolysis becomes activated and free fatty acids (FFA) also known as NEFA are released. **Figure-4B** shows a more in depth look at HSL's function. Phosphorylated HSL's proceed to hydrolyze triglycerides (TG) and diglycerides (DG). Monoglyceride lipase completes lipolysis by cleaving the remaining fatty acid (FA) from the

glycerol backbone. Not shown is that glycerol is also released, not solely NEFAs (Allen et al., 1986; Langin, 2006).

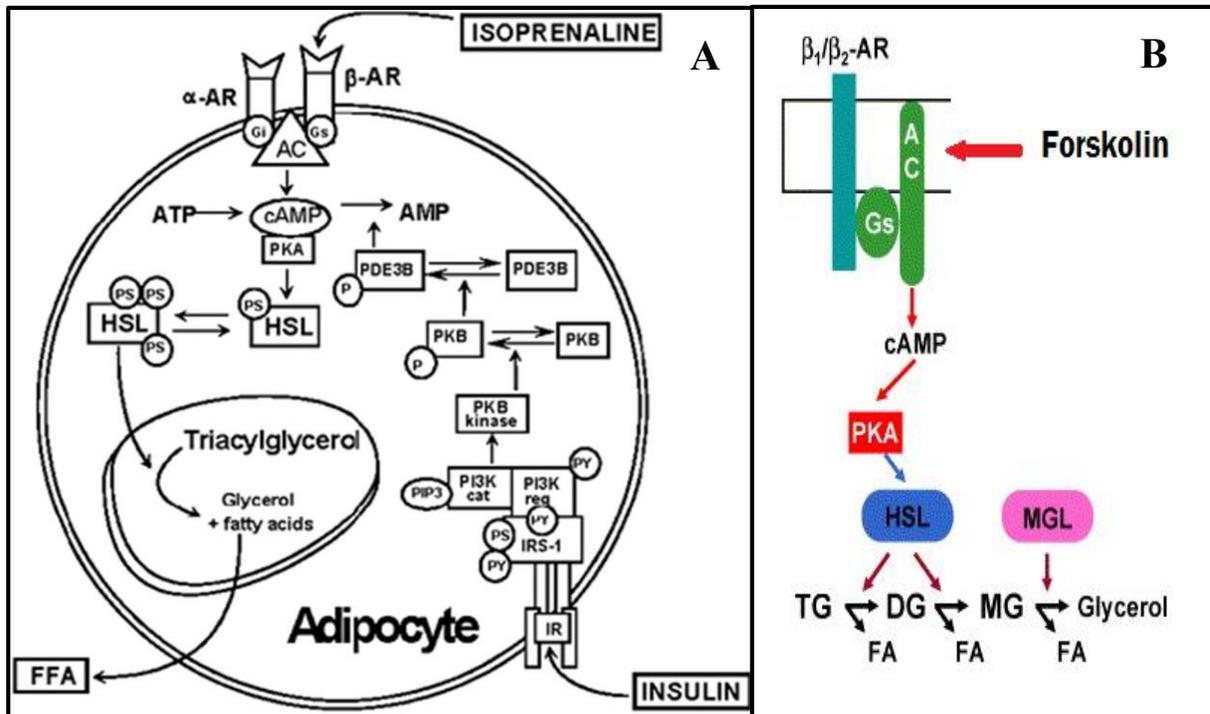


Figure-4: Illustration of the Mechanisms of Isoprenaline (Isopretornol) and Forkolin in Adipocytes. A; Isopretornol stimulates lipolysis by first stimulating β -Adrenergic receptor (β -AR) on the surface of adipocytes. B; Forskolin directly stimulates adenylate cyclase (AC) to produce cyclic AMP (cAMP) that then activates protein kinase A (PKA) to phosphorylate hormone sensitive Lipase (HSL). Phosphorylated HSL proceeds to hydrolyze triglycerides (TG) and diglycerides (DG). Monoglyceride Lipase completes lipolysis by cleaving the remaining fatty acid (FA) from the glycerol backbone and free fatty acids (FFA) are released (Santesson, et al., 2009; Langin, 2006).

Lipolysis can be measured by assaying the levels of local NEFA and glycerol, as they are the products of the process. Although measuring either would provide proof of lipolysis, measuring glycerol would be more accurate as shown earlier in Figure-3, that although NEFA are released from adipocytes, an unknown quantity is recruited back into the cell. These recruited NEFA are reacted with acyl-coA for esterification. Isopretornol and forkoline serve as treatments that can stimulate lipolysis for investigation.

PROJECT PURPOSE

Excessive fat deposition heightens the susceptibility of individuals to disease by disrupting hormone homeostasis that controls proper cellular functions. It is no surprise that \$150 billion dollars is spent each year in the United States to treat obesity-related illnesses (Ferran, 2010). This makes obesity both a major health concern and a major economic burden (Haslam and James, 2005). To better characterize the response of visceral white adipose tissue (WAT) to endocrine stimuli, an ex-vivo model of adipose physiology was developed using primary WAT tissue cultures from DBA/2J mice. This study compared the adipose tissue response from obese mice that had been fasted versus those that were fed regularly. The primary pathways that were stimulated and examined were the β -adrenergic pathway and the direct stimulation of adenylyl cyclase to promote lipolysis.

METHODS

Extraction of Mouse Visceral Adipose Tissue

DBA/2J mice were used in this study as they become obese as adults. As this study called for a specific type of fat tissue, visceral adipose tissue, it was very important to collect the proper adipose tissue from mice as there are two types: subcutaneous and visceral. To keep the collection of tissue consistent, **Figure-5** illustrates the site that the adipose tissue was collected from each mouse. Perirenal fat pads, surrounding the kidneys, and gonadal fat pads, surrounding the sex organs, were collected from each mouse. The fat pads are highlighted in yellow and labeled with red and blue arrows.

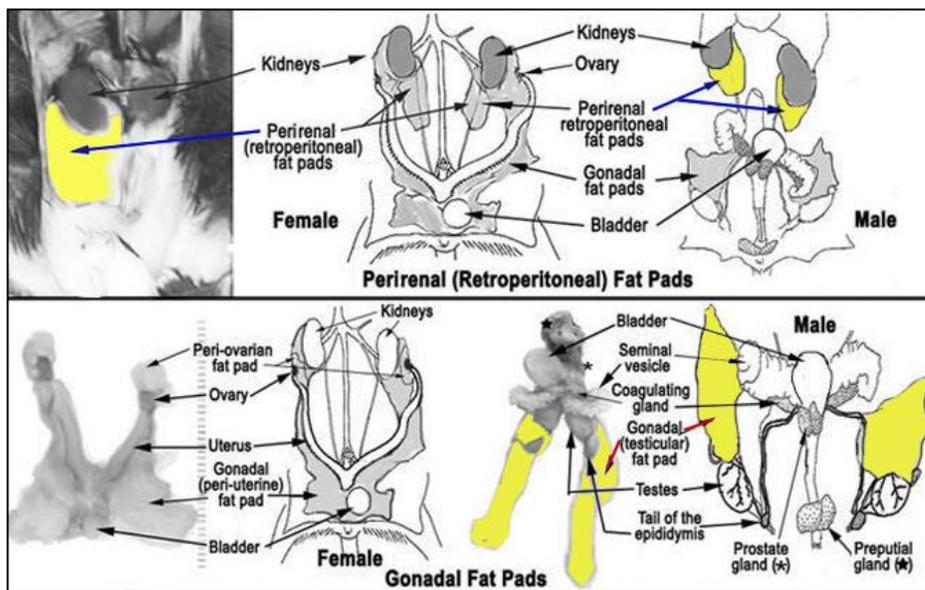


Figure-5: Drawings of the Anterior Inferior Organs of Mice From Which Adipose Tissue was Dissected. Shown is a laboratory mouse with the peri-renal and gonadal fat pads highlighted in yellow. All organs and fat depots are labeled in female (center drawings) and male (right drawings). The diagrams are equated to photographs on the left. (Reed et al., n.d.)

Sex was not a primary focus of this study, therefore the fat pads of both female and male mice were collected, and mixed in one sterile container with PBS Buffer. Feeding regimens were a primary focus for this study, and mice fed on different protocols were separated along with their respective tissues. Fasted mice were fasted 16 hours overnight prior to fat extraction, while control mice were fed regularly. The fat pads were excised, and were cut using laboratory sheers into an eighth of fingernail pad size sections to increase surface area. These cut tissues were then extracted from the PBS buffer, and 3-4 pieces were placed in 24 well culture plates with 300uL of treatment media. One set of studies was done immediately following dissection, while the other set was done 72 hours post dissection.

Adipose Tissue Culture

The purpose of adipose tissue (AT) culture was to treat the perigonadal tissue collected from mice with agents that mimic the effects of catecholamines to stimulate lipolysis. Media was made with Krebs ringer buffer to provide necessary nutrients for the tissue during cultures for 4 and 18 hours. Following each respective time interval 200 uL of medium was extracted from each plate well, collected in micro test tubes, flash frozen, and stored in a -80°C freezer for later assays.

Krebs Ringer Buffer solution was prepared with sodium chloride, potassium chloride, calcium chloride, 10 mM D-glucose, and 5 mM Hepes. For every half liter, 8.6 g, 0.3 gm, and 0.33 gm of each chloride compound, respectively, were added and the pH of the solution was equilibrated to 7.4. PennStrep was also added to the buffer at a 1/100 concentration. The

concentration glucose was chosen to be 10 mM to represent physiological conditions, as glucose concentrations above this amount are considered diabetic. 0.5% BSA was the final concentration used for the investigation of catecholamines to represent physiological conditions by not overly stimulating lipolysis. However, Krebs Ringer Buffer solutions with 0% BSA and 3.0% BSA were also investigated. The results for each of these BSA concentrations are shown in the Results section.

Two concentrations of isoprenalol and forskolin were used in the study alongside controls. Four repetitions of each treatment were done in 24 well plates. The low concentrations of isoprenalol and forskoline used were 4 ug/mL, while the high concentrations used were 12 ug/mL. The samples were incubated at 37°C for 4 hours and then for 18 hours. 100 uL of media was collected from each well at respective time intervals.

Non Esterified Fatty Acid (NEFA) Concentration Assay

Non esterified fatty acids (NEFA) are free fatty acids that have been cleaved by lipase from the glycerol backbone. In stimulating lipolysis through tissue culture, NEFA levels were measured from the media with which the tissues were cultured. The kit used to measure NEFA levels was the Wako Diagnostics NEFA-HR(2) Microtiter procedure. This was a colorimetric assay measured spectrophotometrically at 550nm. The kit comes with Color Reagent-A which is acyl-CoA synthetase (ACS), Color Reagent-B which is acyl-CoA oxidase (ACOD) and peroxidase (POD), and standard NEFA at a concentration of 1.0 mM (Wako Diagnostics, 2013).

This kit is purely enzymatic and is measured through optical densities. Acyl-CoA synthetase reacts with NEFA and forms thiol esters known as acyl-CoA. Color reagent B contains the enzyme that then oxidizes acyl-CoA into hydrogen peroxide. This oxidation allows peroxidase to produce the purple colored end product with the remaining species that can be measured optically at 500 nm. The complete mechanism can be seen in **Figure-6** below (Wako Diagnostics, 2013).

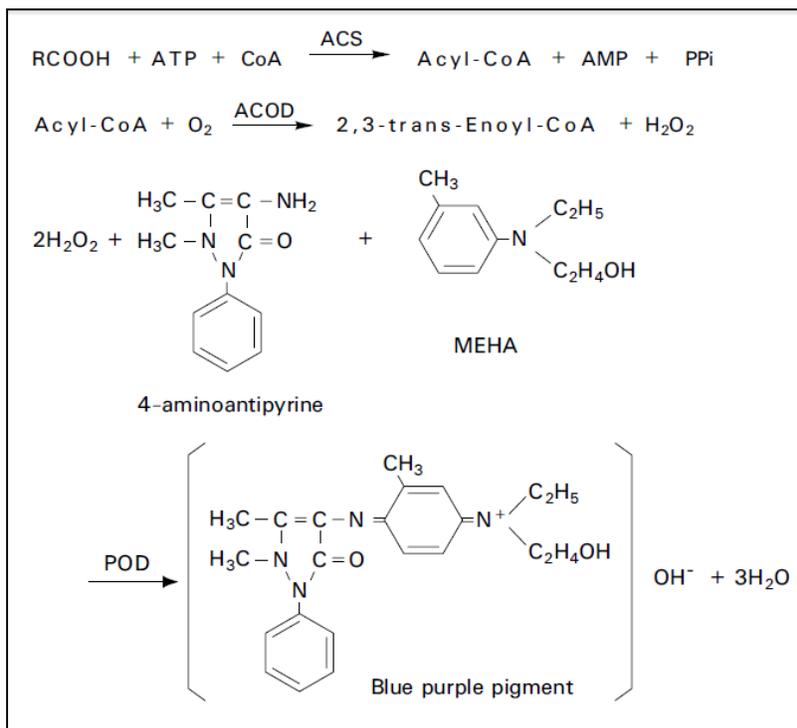


Figure-6: Principle of the Method of NEFA Kit from Wako Diagnostics.

Shown is the illustrated mechanism of the theory behind the Wako NEFA quantitative assay. Abbreviations: Acyl-CoA synthetase (ACS), pyro-phosphate (PPi), Acyl-CoA oxidase (ACOD), Peroxidase (POD) (Wako Diagnostics, 2013).

Standard solutions were made at 1.0 mM, 0.5 mM, 0.25 mM, 0.125 mM and 0.0 mM was made with only de-ionized water with the Wako Diagnostics NEFA Standard Solution kit. The samples and standards were spun on a vortex for 15 seconds then 3uL of each

sample, with duplicate, was pipetted into a 96 well plate alongside the standards, with duplicate, in the same volume. The protocol instructed that 130uL of Color reagent A be pipetted into each sample and standard well. The plate was then covered and agitated in a circular motion on the bench top. Then the plate was incubated for 5 minutes at 37° C and then read at 550 nM spectrophotometrically. Next, 65 uL of Color Reagent-B was added into each occupied well, then the mixing, incubation and reading procedure, was performed as described above. The data was analyzed in Microsoft Excel by first subtracting the Color Reagent-A output values from the respective Color Reagent-B output values, then the samples and standards were averaged with their respective duplicates. An equation for the line of best fit for the standard curve was made, and the optical density value for the blank (0.0 mM standard NEFA) was subtracted from all wells. This equation was used to calculate the concentration of all unknown samples.

Glycerol Concentration Assay

Glycerol is an alcohol that makes up the backbone of triglycerides that is not reused or transported back into the adipocyte, unlike NEFA. Glycerol detection is an accurate measure of lipolysis as it is one of the direct products yielded from the process. A colorimetric assay was performed to detect glycerol species in media using an optimized version of Cayman Chemical's Glycerol Assay Kit (caymanchem.com). Only the color reagent and the standard solution from the kit were used.

The standard solution is composed of free glycerol, and the color reagent is composed of glycerol kinase, glycerol phosphate oxidase, and hydrogen peroxide. Glycerol kinase phosphorylates free glycerol that is then oxidized by glycerol phosphate oxidase producing

hydrogen peroxide. Peroxidase then reacts with hydrogen peroxide and other species to produce a purple product that can be measured spectrophotometrically at 540 nm (Caymen Chemical Company, 2013).

The standards were made at concentrations of 0.025 mg/mL, 0.0125 mg/mL, 0.00625 mg/mL, 0.0025 mg/mL, and 0.00125 mg/mL, with de-ionized water used as a blank. To measure the level of glycerol in the media, 20 uL of samples with duplicates and standards with duplicates were micropipetted into a 96 well plate. The color reagent at a volume of 80 uL was then added to each occupied well. The plate was then covered and agitated for 30 seconds in a circular motion on the bench top, and then placed in an incubator set at 37°C for 5 minutes. The optical density of the plate was read at 540nm and analyzed in Microsoft Excel. The samples and standard duplicates were averaged, respectively, and the blank readings were then subtracted from all wells to ensure as much background as possible was removed from wells for proper analysis. An equation for the line of best fit for the standard curve was then made, and this equation was used to calculate the concentration of all unknown samples.

RESULTS

Previous research found that adipose tissue lipolysis increases during fasting. To study this process further, fasted (F) and not fasted (NF) obese mouse adipose tissue were cultured in Krebs ringer buffer for 4 hours and 18 hours, then the levels of lipolysis were measured by assaying the local levels of nonesterified fatty acids (NEFA) and glycerol released into the media. The mean of the NEFA data is shown in **Figure-7**. The F-mouse adipose tissue regardless of culture time had significantly more ($p < 0.01^{**}$) local levels of NEFA than NF-mouse adipose tissue. Thus, fasting elevates lipolysis in adipose tissue as measured by NEFA. The student t-test was done with Microsoft Excel to test the significance of these findings. Comparing the 4 hour culture of F-mice tissue to NF-mice through the student t-test a p-value of $3.01E-7$ was observed. For the 18 hour culture, a p-value of $5.44E-7$ was observed.

The mechanism of lipolysis in conjunction with hormone homeostasis in visceral white adipose tissue (WAT) is not fully understood. To investigate this, WAT from obese mice were treated with two hormones known to stimulate lipolysis through different mechanisms. Isoproterenol (Ispr) stimulates lipolysis by stimulating the β -Adrenergic receptor (β -AR) on the surface of adipocytes. Forskolin (Frk) directly stimulates adenylate cyclase (AC) to produce cyclic AMP (cAMP) which then activates protein kinase A (PKA) to phosphorylate hormone sensitive Lipase (HSL).

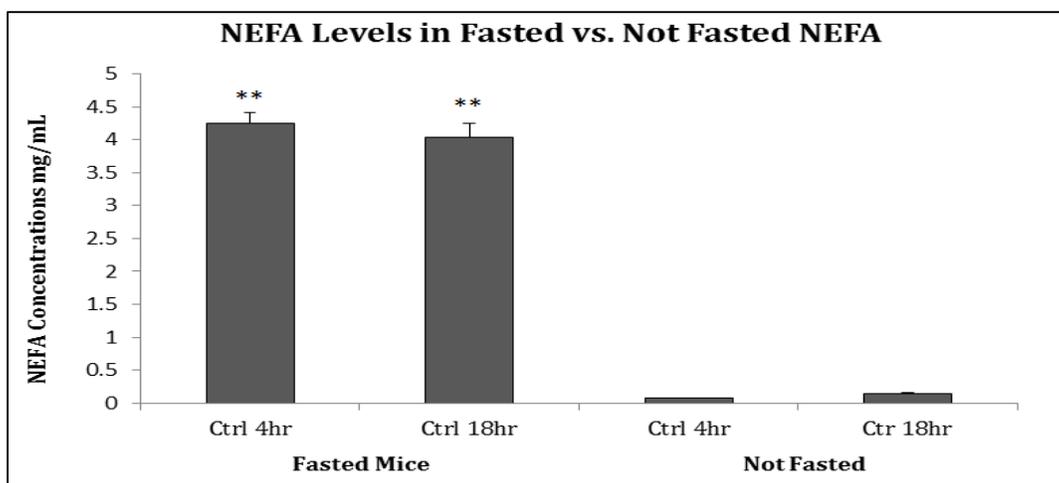


Figure-7: Lipolysis Measured by NEFA In the Media for Fasted and Not Fasted Adipose Tissue from Obese Mice. Statistical significance on the graph is denoted by a singular asterisk ($p < 0.05$ *), and statistically highly significance ($p < 0.01$ **) is denoted by two asterisks. Fasted data are on the left. Not fasted data on the right.

Tissues from mice were treated with isoprenalol or forskolin at concentrations known previously to stimulate lipolysis. Isoprenalol concentrations used were 4 $\mu\text{g/mL}$ and 12 $\mu\text{g/mL}$, and the Frk concentration used was 4.1 $\mu\text{g/mL}$ or 10 μM . The NEFA data for isoprenalol are shown in **Figure-8**. Once again, it is visually apparent that F-mouse adipose tissues, whether stimulated with isoprenalol or not, yield a greater lipolytic response than NF. The NEFA comparison between F-and NF- treated with isoprenalol (right histobars) has a p-value of 4.82E-7, so fasting significantly induces lipolysis even after stimulating lipolysis through the β -adrenergic receptor. Comparing NF-control adipose to NF-adipose treated with isoprenalol a p-value of 2.19E-6 was observed, showing that isoprenalol did in fact stimulate lipolysis. Isoprenalol also induced lipolysis in the fasted samples, a p-value of 0.0024 was observed.

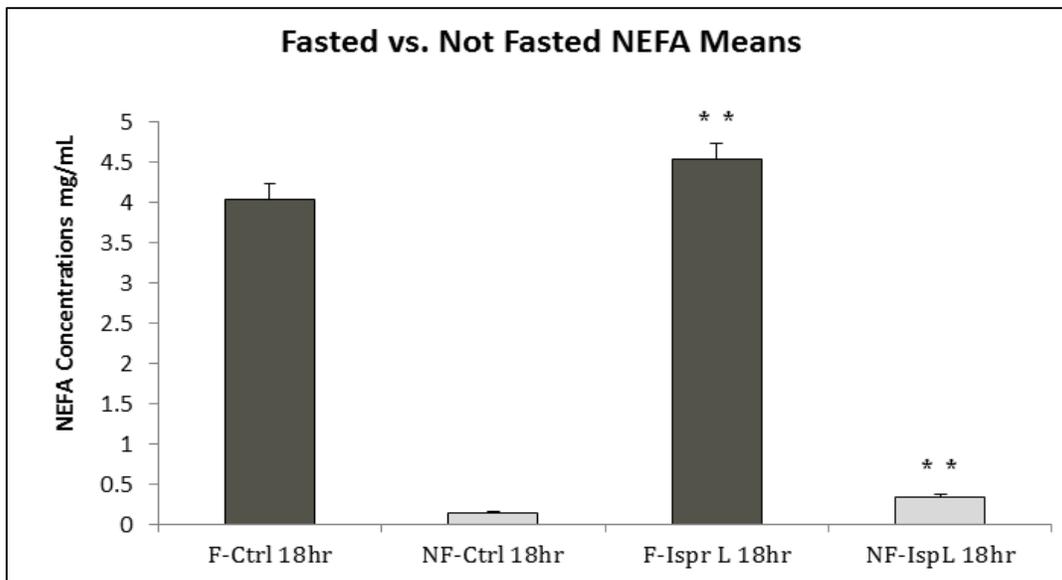


Figure-8: Media NEFA Levels in Isopretornol Treated Fasted and Non-Fasted Adipose. Statistical significance on the graph is denoted by a singular asterisk ($p < 0.05$ *) or highly significant ($p < 0.01$ **) with two asterisks. Fasted (F), not fasted (NF), control (Ctrl), isopretornol (Ispr).

The NEFA forskolin data are shown in **Figure-9A**. Forskolin did not appear to significantly induce or dampen lipolysis after 4 hours or after 18 hours of culture. The p-values observed were 0.25 and 0.45 respectively, which are >0.05 so not statistically significant. A repeat of the isopretornol experiments showed that while 18 hours of culture yielded a statistically significant increase in lipolysis (p-value of 0.0024), low concentrations of isopretornol after 4 hours of culture failed to significantly stimulate lipolysis (p-value of 0.065). Treatment with a high concentration of isopretornol stimulated lipolysis significantly following 18 hours of culture (p-value 0.039), but not at 4 hours of culture (p-value 0.055).

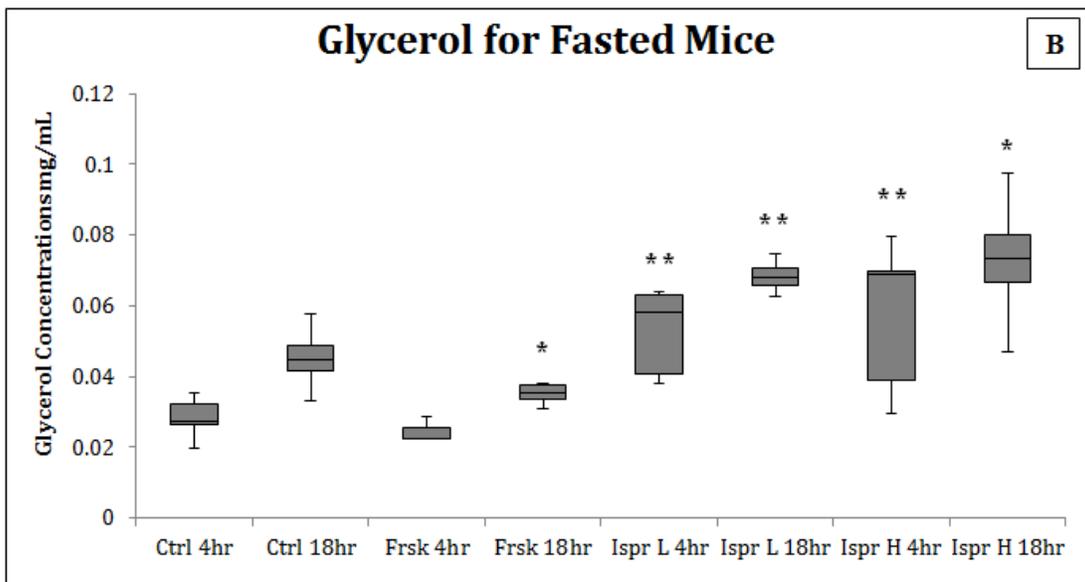
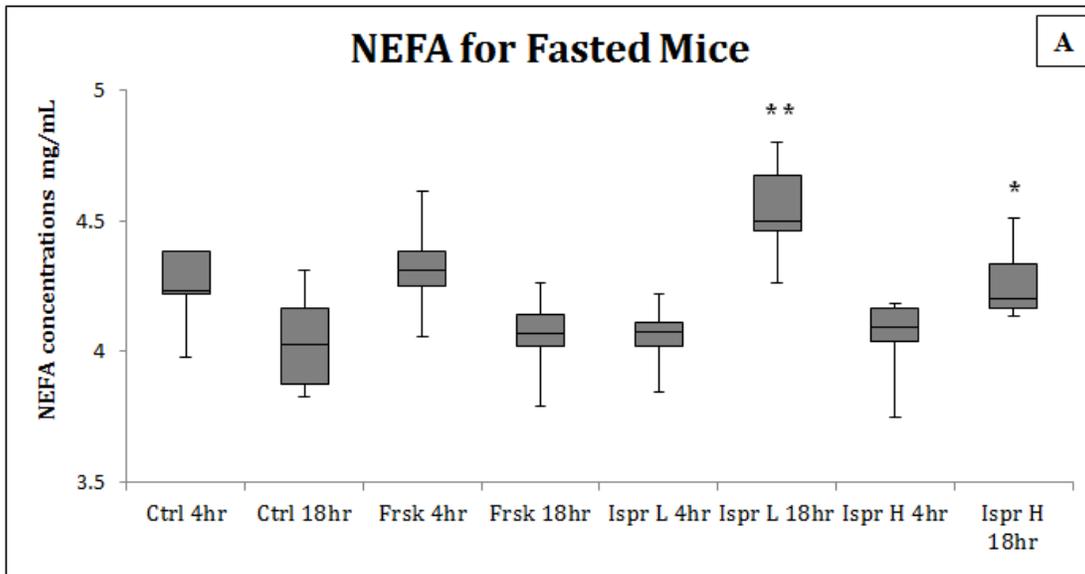


Figure-9: Comparison of the Lipolytic Stimulation As Measured by NEFA and Glycerol Media Concentrations, With and Without Forskolin and Isoprenalol. Statistical significance on the graph is denoted by a singular asterisk ($p < 0.05$ *) or highly significant ($p < 0.01$ **). Forskolin (Frsk), Isoprenalol (Ispr), Control (Ctrl).

Lipolysis was also measured through local glycerol levels in the media (**Figure-9B**).

Once again, culture with forskolin for only 4 hours did not stimulate lipolysis relative to

control (p-value 0.112), but 18 hours of culture proved to significantly stimulate lipolysis (p-value 0.033). Both low and high concentrations of isoprenalol cultured for 4 hours proved to significantly stimulate lipolysis, p-values of 0.004 and 0.019, respectively. Both low and high concentrations of isoprenalol significantly increased glycerol after 18 hours of culture (p-values 0.0012 and 0.033, respectively). Since both forskolin and isoprenalol treatments significantly increased local levels of glycerol in the media following 18 hours of culture, a comparison was done between the two at similar treatment concentrations, 4 ug/ml of isoprenalol and 4.1 ug/ml of forskolin. A p-value of 6.46E-5 was observed, indicating the two treatments differed significantly. Together, the glycerol and NEFA data confirm that directly stimulating the β -adrenergic cell surface receptor with isoprenalol yields a greater lipolytic response than does directly stimulating adenylyl cyclase with forskolin.

Krebs Ringer Buffer and DMEM are two popular tissue and cell culture media. For most of this MQP, Krebs Ringer Buffer was used. To determine whether the two types of media differ in the lipolysis experiments, the two media were compared (**Figure-10A**). The two media did not differ significantly in their NEFA levels (light grey versus dark grey histobars) in the presence of increasing concentrations of BSA. BSA was tested because previous studies showed it is used to transport NEFA from lipid droplets. 0.5% BSA yielded an insignificant p-value of 0.53 relative to control, while 3.0% BSA yielded a significant p-value of 0.004. To prevent any significant effects of BSA in the culture media on lipolysis, 0.5% BSA was used throughout the experiments.

In order to determine whether glucose concentrations in the media had an impact on lipolysis, a study was done at 0 uM, 5 uM, 10 uM, and 25 uM glucose in 0.5% BSA (dark grey histobars) and 1.0% BSA (light grey histobars) (**Figure-10B**). NEFA levels did not vary

significantly at different glucose concentrations, although the BSA had an impact. These findings show that the glucose and BSA conditions used in this study did not significantly affect lipolysis, thus the study was valid and the levels were affected by the drug treatments and the fasting.

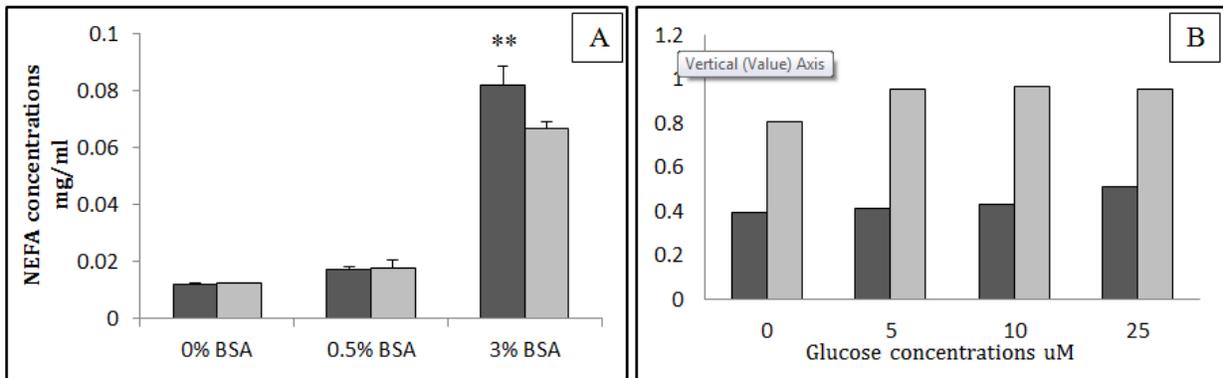


Figure-10: NEFA Levels in the Media at Various BSA and Glucose Concentrations. Panel-A: DMEM is depicted as light grey while Krebs Buffer is depicted as dark grey. In Panel-B, 1.0% BSA is depicted as light grey, while 0.5% BSA is depicted as dark grey.

DISCUSSION

This MQP investigated the effects of fasting and two different drugs on lipolysis in primary adipocyte cultures from mice. Adipose tissue explants were dissected from the visceral depots of fasted and un-fasted mice, and then cultured. Visceral fat was chosen because mice with a great deal of fat stored in the subcutaneous regions tend to have healthier smaller adipocytes, although the mice are obese. Media from ex-vivo cultures were collected at 4 and 18 hour time intervals, and the concentrations of released non-esterified fatty acids (NEFA) (a product of the breakdown of triglycerides) or glycerol were measured in the media as an evaluation of lipolysis.

Adipose tissue is the main energy depot in humans and mice. During fasting, triglycerides stored in lipid droplets of adipocytes are hydrolyzed to release NEFAs. An unknown portion of these NEFA are oxidized by other tissues and used as fuel, while the remaining portion is recruited back into the cell for re-esterification. However, NEFA levels are still a reliable measure of lipolysis. In this study, the data showed that lipolysis significantly increased in adipose tissue following fasting.

With the recent discoveries of adipocyte-derived hormones, such as adiponectin, the regulatory role of endocrine effects on lipid metabolism has surfaced as an important system for maintaining energy homeostasis. Conditions that yield changes in the metabolic state of adipocytes, such as the American epidemic of obesity, have been linked to disorders in the endocrine function of visceral white adipose tissue (WAT). To better characterize this lipolytic response of WAT to endocrine stimuli, our ex-vivo model of adipose physiology was tested using primary tissue culture on DBA/2J mouse adipose treated with two types of

endocrine stimuli, isoprenalol (4 ug/mL and 12 ug/mL) which increases lipolysis by directly activating the β -adrenergic receptor, and forskolin (10 uM or 4.1 ug/mL) which increases lipolysis by directly activating adenylyl cyclase (AC). The two different mechanisms of lipolytic stimulation were compared at similar concentrations, and the data indicated that direct stimulation of the β -adrenergic receptor significantly increased lipolysis in fasted mouse WAT relative to untreated fasted controls, while stimulating adenylyl cyclase did not. This was the case whether monitoring NEFA or glycerol. Because excreted glycerol is not recruited back into adipocytes, it may provide a more accurate measure of lipolysis than NEFA which can be recruited back inside cells. Together, our data show that endocrine stimuli can play a major role in the lipolytic response of visceral WAT in both fasted and fed mice.

Future research should be aimed at confirming the role of phosphorylated P38 mitogen-activated protein kinase (PP38) in lipolysis, because obesity puts stress on tissues. PP38 affects calcium concentration which also affects many cellular functions. Thus, continued efforts should also focus on further characterizing the pathways that affect lipolysis in WAT, with special interest on the effects of calcium.

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