

INVOLVEMENT OF PFKFB3/iPKF2 IN THE EFFECTS OF
LEUCINE AND n-3 PUFA IN ADIPOCYTES

A Thesis

by

VERA HALIM

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

December 2011

Major Subject: Nutrition

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Approved by:

Chair of Committee,	Chaodong Wu
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ABSTRACT

Involvement of PFKFB3/iPFK2 in the Effects of Leucine and n-3 PUFA in Adipocytes.

(December 2011)

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Chair of Advisory Committee: Dr. Chaodong Wu

Studies had shown that leucine supplementation increases insulin sensitivity and it has been studied that n-3 PUFA may have an anti-inflammatory effect in adipocytes. However, the extent to which dietary sources such as leucine and/or n-3 PUFA act through PFKFB3/iPFK2 to suppress adipocyte inflammatory response has not been studied; PFKFB3/iPFK2 is a regulator that links adipocyte metabolism and inflammatory responses. In this study, the involvement of PFKFB3/iPFK2 in the effects of insulin sensitizing and anti-inflammatory effect of leucine and/or n-3 PUFA are explored using cultured 3T3-L1 adipocytes including wild-type cells, PFKFB3-control cells (iPFK2-Ctrl) and PFKFB3-knockdown cells (iPFK2-KD).

In iPFK2-Ctrl cells, leucine supplementation appears to have insulin-sensitizing effects through improving p-Akt/Akt insulin signaling, but have no effect on adiponectin expression, and appear to have limited anti-inflammatory effects. n-3 PUFA supplementation appears to have limited effects on both insulin sensitizing and anti-inflammatory effects in iPFK2-Ctrl. In contrast, n-3 PUFA exhibit pro-inflammatory expression in iPFK2-KD. The results of this study support the hypothesis that

PFKFB3/iPFK2 is critically involved in insulin-sensitizing effects of leucine. This role of PFKFB3/iPFK2, however, appears to be independent of anti-inflammatory responses. Given this, it is likely that PFKFB3/iPFK2 only account, in part, for the beneficial effects of leucine. n-3 PUFA stimulate PFKFB3/iPFK2 activity in wild-type adipocytes. However, PUFA do not exhibit anti-inflammatory and insulin-sensitizing effects in controls. In contrast, n3-PUFA exhibit proinflammatory effects in iPFK2-KD cells. Taken together, PFKFB3/iPFK2 is involved, at least in part, in the effects of insulin sensitization of leucine and appears to protect adipocytes from inflammatory responses, which could be exacerbated by n-3 PUFA when PFKFB3/iPFK2 is disrupted.

DEDICATION

To the love of my life –

Papa, Mama and Koko

and

To my best friend for life –

Ge

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Wu, for giving me the opportunity to work in his lab and to earn my degree, and for his continuous support, patience and guidance throughout my time in graduate school. Thanks also to my committee members, Dr. Chapkin and Dr. Zhou, for their time, support and guidance for this research.

Thanks to Dr. Beiyan Zhou for letting me work in her lab and her time in guiding me with qPCR.

My gratitude to my lab colleagues – Honggui Li, Xin Guo, Xu Hang and Meng Cong for teaching me how to do experiments, for listening, for supporting me and for their friendships.

Thank you to my motivators: my parents, Tedjasurya Halim and Julianty Pradono, my brother, Sugiri, and my best friend, William for their unconditional love, support, endless encouragement, patience, and for believing in me; and to Phoenix, for always being there for me.

Thank you to all of my family and good friends for the great times in the U.S.: to Imei, Getta, Sherly and Vinny for their support and understanding, and to God for everything.

NOMENCLATURE

Ala	Alanine
ALA	α -linolenic Acid
AMPK	5' Adenosine Monophosphate-activated Protein Kinase
BCAA	Branched-chain Amino Acid
BMI	Body Mass Index
C/EBP α	CCAAT-enhancer-binding Proteins
Ctrl	Control
DHA	Docosahexaenoic Acid
DMEM	Dulbecco's Modified Eagle's Medium
EPA	Eicosapentaenoic Acid
ER	Endoplasmic Reticulum
F2,6BP	Fructose 2,6-bisphosphate
FFA	Free Fatty Acids
GTT	Glucose Tolerance Test
HFD	High Fat Diet
IL-6	Interleukin-6
IL-10	Interleukin-10
IKK	Inhibitor of NF κ B Kinase
INS	Insulin
iPFK2	Inducible 6-phosphofructo-2-kinase

IR	Insulin Resistance
IRS	Insulin Receptor Substrate
JNK	c-Jun N-terminal kinase
KD	Knockdown
LA	Linoleic Acid
Leu	Leucine
mTOR	Mammalian Target of Rapamycin
MUFA	Monounsaturated Fatty Acid
NF κ B	Nuclear Factor κ B
Pal	Palmitate
PBS	Phosphate-Buffered Saline
PFK-1	Phosphofructokinase-1
PFK-2/FBPase	Phosphofructokinase-2/fructose-2,6-bisphosphatase
PPAR	Peroxisome Proliferator-activated Receptor
PUFA	Polyunsaturated Fatty Acids
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SFA	Saturated Fatty Acid
T2DM	Type 2 Diabetes Mellitus
TG	Triglycerides
TNF α	Tumor Necrosis Factor α

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1. INTRODUCTION

Adipose tissue is an endocrine organ that manages body's energy reserves, and also synthesizes and secretes adipokines (1). Inflammatory response resides predominantly in adipose tissue. Thus, the biology of adipose tissue has become one of the main focuses on studying the inflammation.

The association between obesity and low-grade inflammatory reaction in the adipose tissue as it contributes to T2DM, cardiovascular disease, hepatic steatosis, airway disease, neurodegeneration, biliary disease and certain cancers (2) has been well documented. Obesity is a major health concern in the world; in the year of 2007-2008, 68% of the U.S. populations ages 20 years and above are either overweight ($\text{BMI} \geq 25 \text{ kg/m}^2$) or obese ($\text{BMI} \geq 30 \text{ kg/m}^2$) (3). The rate of obesity has been increasing and predicted to be increased more in the future.

Mechanistically, an increase in ROS due to excess fatty acid oxidation activates NF κ B and/or JNK pathways, which in turn brings about an increase in adipocyte inflammatory response. Increased of adipocyte inflammation would lead to inappropriate expression of adipokines and decreased in insulin signaling, which contributes to insulin resistance (4). Adipose tissue inflammation is characterized by an increase in adipose expression of proinflammatory cytokines such as IL-6 and TNF α

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and a decrease in the expression of anti-inflammatory cytokines such as IL-10 (5).

PFKFB3 is one of the four genes that encode PFK-2/FBPase. PFK-2/FBPase is an enzyme that synthesizes and degrades F2,6BP, a potent activator of PFK-1, which is the rate limiting enzyme for glycolysis; PFKFB3 codes inducible isoform of 6-phosphofructo-2-kinase (iPFK2) (6). PFKFB3/iPFK2 is the regulator of the adipocyte inflammatory response and it is found mostly in the adipose tissue. It has been recently shown that PFKFB3 disruption exacerbates overnutrition-induced adipose tissue inflammation (6).

Overnutrition causes excessive fatty acids oxidation in adipose tissue, thereby resulting in an increase in inflammatory response. The presence of PFKFB3/iPFK2 is expected to increase the production of glycerol-3-phosphate and FFA by glycolysis and pyruvate oxidation pathway. This appears to channel FFA to triglyceride synthesis, which in turns reduces the fatty acid oxidation-associated production of ROS. Ultimately, a decrease in ROS production protects against overnutrition-induced activation of inflammatory signaling pathways of JNK and NFκB, and suppresses the expression of proinflammatory cytokines (7).

Leucine is an essential branched chain amino acid, which cannot be reproduced and can only be obtained from diet (8). Some example of leucine sources are legumes, whole grains and animal products. Leucine is also a functional amino acid (8) because it is known to be a potent activator of the mTOR (9).

Many researchers have been studied the effects of leucine on glucose metabolism. Studies found that leucine supplementation restores glucose-induced insulin

secretion in protein-malnourished rats (10). Studies had also found that leucine supplementation decreases adipose tissue inflammation and improves glycemic control in HFD-fed mice (9) and mouse models of obesity and diabetes (11).

PUFA is dietary essential fatty acid because it prevents deficiency symptoms, but it cannot be synthesized by humans and it can only be obtain from diet. There are two main families of PUFA: n-3 (α -linolenic acid) and n-6 (linoleic acid). This thesis focuses on docosahexaenoic acid [DHA; 22:6 (n-3)], which found in fish and marine oils (12).

In cultured 3T3-L1 adipocytes, palmitate (saturated fatty acid) caused a decrease in insulin-induced Akt phosphorylation (13), demonstrating a role for overnutrition in inducing adipocyte insulin resistance. Palmitate had also been shown to increase inflammatory responses, such as TNF α , NF κ B, IL-6, ROS, and JNK and decreases anti-inflammatory cytokine (IL-10) in 3T3-L1 adipocytes (13). On the other hand, DHA had been shown to suppress inflammatory responses (NF κ B) and to increase anti-inflammatory cytokines (IL-10) in 3T3-L1 adipocytes (13). In HFD-fed mice, n-3 PUFA efficiently prevented development of obesity and of impaired glucose tolerance (14).

This thesis is divided into four parts: literature review, materials and methods, results, summary and conclusion. The hypothesis of this study is that PFKFB3/iPKF2 is involved in the effects of leucine and/or n-3 PUFA on suppression of overnutrition-induced insulin resistance and inflammatory response in adipocyte.

2. LITERATURE REVIEW

2.1 Adipose tissue inflammation and gene expression

There are two types of inflammation: classic inflammation and metabolic inflammation. Classic inflammation happens when body responses to injuries; symptoms include swelling, redness, pain, fever, and loss of function (15). These are often short-term adaptive responses of the body to deal with injuries for regeneration or repair of the affected tissue which involves integration of signals in cells and organs (15). On the other hand, metabolic inflammation, which sometimes referred to chronic low-grade inflammation, contributes to systemic metabolic dysfunction which associated with obesity-linked disorders (16).

In the year of 2007-2008, in the U.S., 68% of adults age 20 years and over are either obese ($\text{BMI} > 30 \text{ kg/m}^2$) or overweight ($\text{BMI} > 25 \text{ kg/m}^2$) (3), and the number are predicted to be increased more in the future. For more than a decade, obesity has become an epidemic and has been recognized as a disease. Obesity is the most important factor in the increasing incidence of metabolic diseases, because it causes low-grade inflammatory reaction particularly in white adipose tissue, which contributes to T2DM, cardiovascular disease, hepatic steatosis, airway disease, neurodegeneration, biliary disease and certain cancers (2). It is characterized by adipose tissue growth, where both adipocytes hypertrophy (increase in size) and hyperplasia (new adipocytes formed) occurs (17).

Adipose tissue is an endocrine organ that synthesizes and secretes adipokines, which consist of pro-inflammatory or anti-inflammatory cytokines (2). Currently, there are more than 50 adipokines have been discovered; leptin and adiponectin are the two adipokines that are most studied. Leptin is an adipocyte hormone which mainly made and secreted by mature adipocytes and binds to its receptor in the hypothalamus (18); it regulates appetite, body weight, food intake and energy expenditure. Elevated plasma leptin results in weight gain (19). Adiponectin, on the other hand, plays an important role in regulating insulin sensitivity and energy homeostasis by protecting against inflammation and obesity-linked insulin resistance (20).

Studies had shown that adipose tissue dysfunction such as adipose tissue inflammation, rather than excess adipose tissue mass that leads to development of insulin resistance, the mediator of obesity-related morbidity (2). Thus, biology of adipose tissue has become the main focus in the study of these metabolic diseases.

3T3-L1 and 3T3-F442A cell lines are the most frequently used in the study of adipocytes (18); 3T3-L1 cell line derived from mouse fibroblast 3T3 (21). Adipocytes evolved from undifferentiated fibroblast-like preadipocytes into mature adipocytes (17). Adipocyte differentiation converts the preadipocyte to adipocyte where they obtained their spherical shape, lipid droplets, and has similar structure and biochemical characteristics of a mature white adipocyte (18).

There are set of gene-expression events involve in adipocyte differentiation, in which the PPAR γ and C/EBP α play an important role as transcription factors. PPAR γ is a member of nuclear-receptor superfamily; it is the master regulator of adipogenesis.

Thus, it is required for induction and maintenance of adipocyte differentiation in fibroblasts (22). C/EBP α induced after the induction of PPAR γ during adipogenesis. High levels of C/EBP α in fibroblastic cell also required for induction into adipocyte cells. C/EBP α and PPAR γ works together to achieve and maintain differentiated state of adipocyte (23).

Insulin binds to growth factor receptors to phosphorylate and activate Akt, once Akt is activated; glucose goes into the cells through GLUT4 transporter. Glucose goes through glycolysis to produce pyruvate before it gets into mitochondria for TCA cycle. During TCA cycle, nutrients were oxidized, electrons were donated to electron transport chain, and ATPs were produced. Incomplete reduction of oxygen (O_2) produces superoxide (O_2^-), which is a reactive oxygen species (ROS). O_2^- can convert into more stable form which is hydrogen peroxide (H_2O_2), then to hydroxyl radical (OH^\cdot). ROS produced at low level by electron transport chain as normal part of cellular metabolism, and it is important for regulation of cell signaling, proliferation and differentiation. However, during the condition of overnutrition such as obesity, ROS production exceeds what is needed for cells to maintain its normal function and this can lead to ER stress, which activates the inflammatory pathway (24), as shown in FIGURE 1.

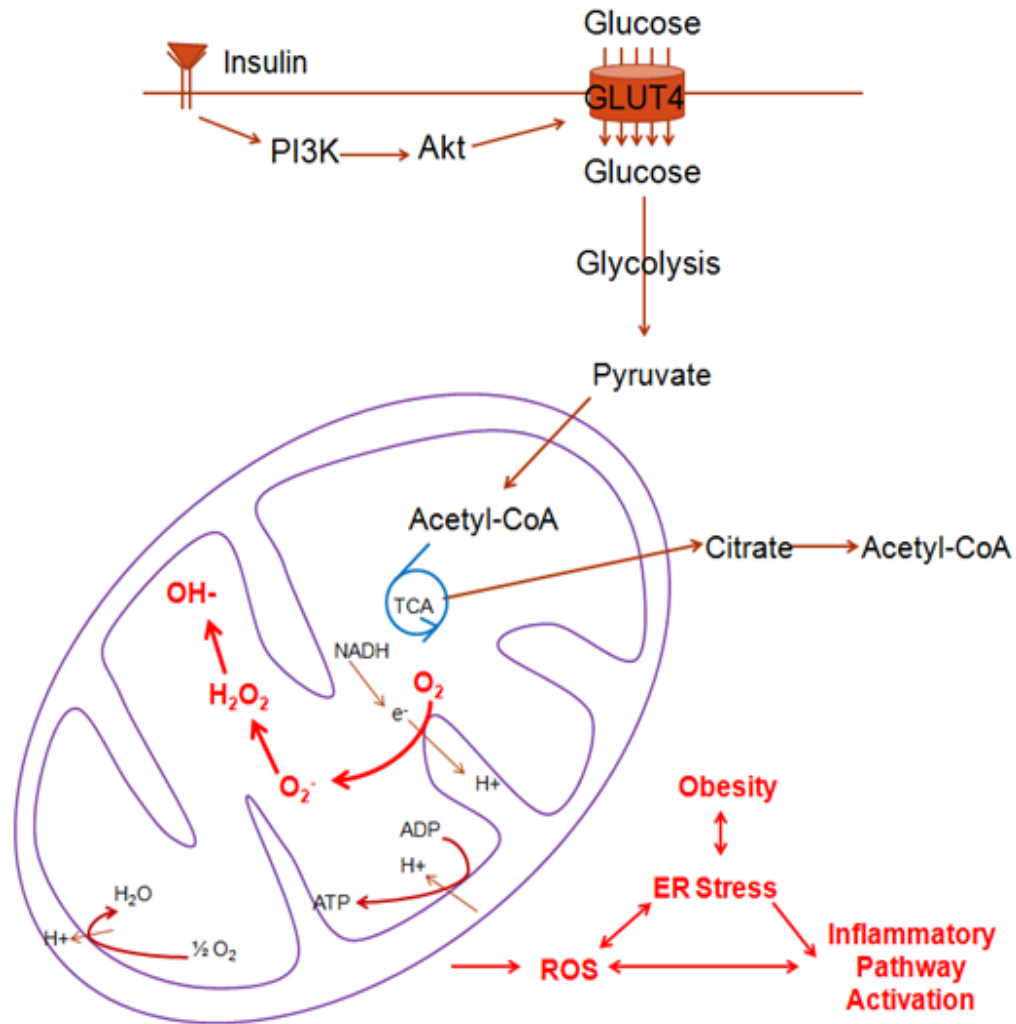


FIGURE 1. Overproduction of ROS leads to inflammatory pathway activation. Modified based on Wellen, et al. (24).

Adipose tissue inflammation is characterized by activation of inflammatory signaling pathways, abnormal cytokine production, and increased acute-phase reactants, i.e., an increase in adipose expression of proinflammatory cytokines such as IL-6 and TNF α and a decrease in the expression of anti-inflammatory cytokines such as IL-10 (5). Overproduction of proinflammatory cytokines stimulate the JNK and IKK- β /NF κ B pathways to upregulate potential mediators of inflammation which lead to insulin resistance (25).

2.2 Obesity, insulin resistance and diabetes

ER is a critical organelle responsible for the synthesis, maturation, folding and transport of all secreted and transmembrane proteins. It is also the site for lipid synthesis and packaging. In the case of obesity, adipose tissue dysfunction happens when elevated ROS production and functional capacity of ER is overloaded (26-28); this induces the activation of inflammatory signaling pathway and leads to activation of serine/threonine kinases by inflammatory or stressful stimuli which contribute to stimulation both JNK and IKK- β /NF κ B pathways, resulting in upregulation of mediators of inflammation (29). Thus, in insulin resistance condition, JNK and NF κ B activities are elevated and in studies with both genetic and dietary mouse models of obesity, absence of JNK1 prevents development of insulin resistance and diabetes (16).

Insulin works by binding to its receptor on the surface of insulin-responsive cells. Insulin resistance is a physiological condition where target cells become less sensitive to insulin. Insulin resistance develops when the insulin receptor IRS proteins

phosphorylated and thus inhibits their function and interferes with insulin signaling. The primary mechanism of insulin resistance is when $\text{TNF}\alpha$ or elevated levels of FFAs stimulate cells to induce inhibitory phosphorylation of serine residues of IRS-1. In turn, this inhibits downstream insulin signaling and insulin action which leads to insulin resistance by reduces both tyrosine phosphorylation of IRS-1 in response to insulin and the ability of IRS-1 to associate with the insulin receptor (30), as shown in FIGURE 2.

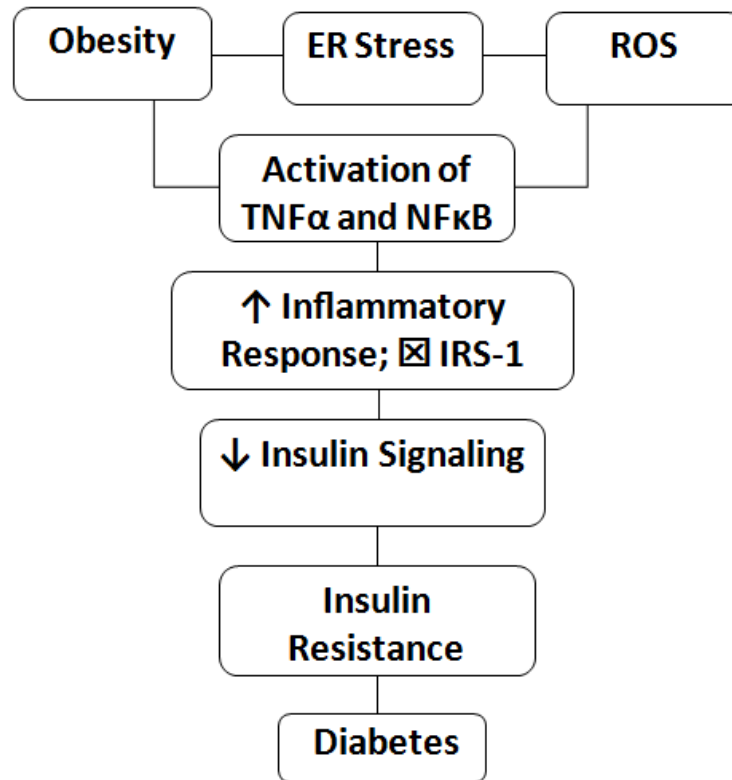


FIGURE 2. Relationship between obesity, inflammation and metabolic diseases. Obesity leads to inflammation, which is the primary cause of metabolic disease rather than the consequences of obesity itself. Adapted and modified based on Wellen, et al. (16).

Type 2 diabetes is an inflammatory disease with insulin resistance. The mechanisms are the same as insulin resistance, hyperglycemia stimulates ROS production in adipocytes, and in turn, it increases the production of proinflammatory cytokines. On the other hand, adiponectin is a protein hormone that acts as an insulin sensitizer that stimulates fatty acid oxidation in AMPK and PPAR α . In insulin resistance condition, level of adiponectin is decreased (25) and characterized by macrophage infiltration in adipose tissue (31).

2.3 PFKFB3/iPFK2 and adipocyte physiology

PFKFB3 is one of the four genes (PFKFB1, PFKB2, PFKFB3 and PFKFB4) that encode PFK-2/FBPase. PFK-2/FBPase is an enzyme that synthesizes and degrades fructose 2,6-bisphosphate (F2,6BP), a potent activator of PFK-1, which is the rate limiting enzyme for glycolysis (32); PFKFB3 codes inducible isoform of 6-phosphofructo-2-kinase (iPFK2) (6). PFKFB3/iPFK2 is the regulator of the adipocyte inflammatory response and it is found mostly in the adipose tissue (6) and phosphorylated by AMPK on Ser461 (32). It has been recently shown that PFKFB3 disruption exacerbates overnutrition-induced adipose tissue inflammation (6).

In the glycolysis pathway, the presence of PFKFB3/iPFK2 enhances the production of 6PFK1, which in turns enhances the glycolysis pathway. Diet also provides cells with FFA which get into the cells through fatty acid transporters. Glycerol-3-phosphate and FFA, as the two main products of nutrient metabolisms, convert into triglycerides for storage in adipocytes (7), as shown in FIGURE 3.

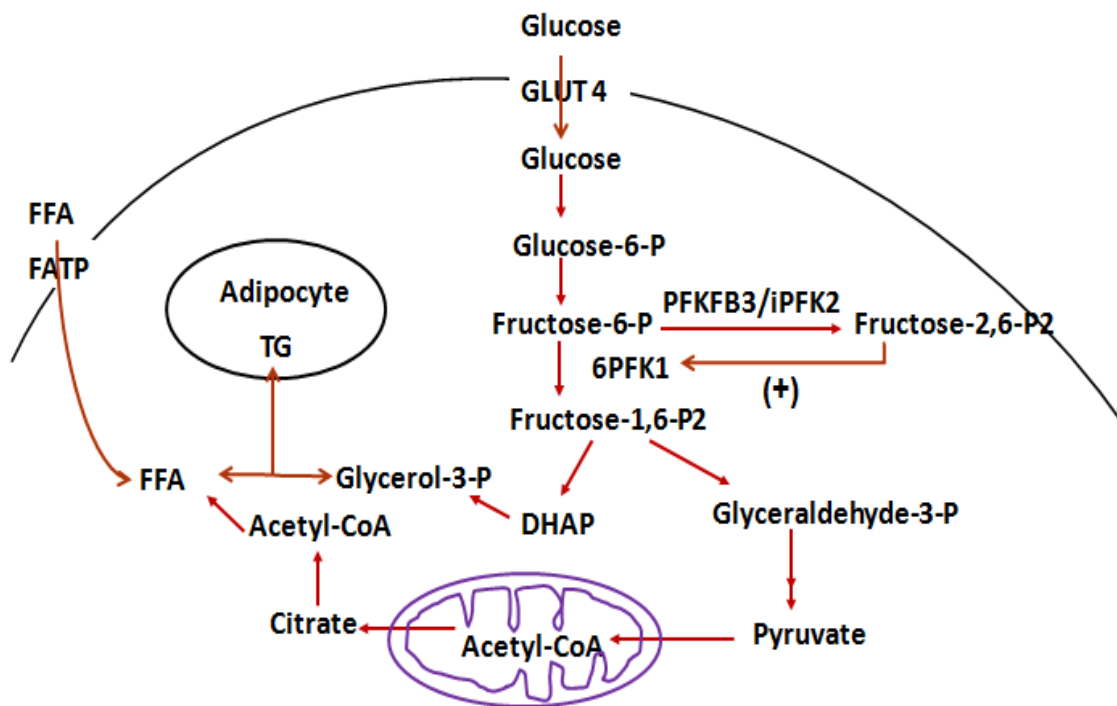


FIGURE 3. **PFKFB3/iPFK2 enhances 6PFK1 enzyme production.** Modified based on Guo, et al. (7).

Without the presence of PFKFB3/iPFK2, 6PFK1 enzyme is less active, which leads to suppression of glycolysis pathway. This, in turns, produces fewer products such as DHAP and glyceraldehyde-3-phosphate, as well as glycerol-3-phosphate and FFA; however, cells still obtain FFA from dietary sources. Consequently, storage of triglycerides is suppressed and extra FFA in cells lead to increased fatty acid oxidation and ROS production. This activates inflammatory pathways such as JNK1 and NFκB pathways, and in turns, lead to increasing in proinflammatory cytokine expression (7), as shown in FIGURE 4.

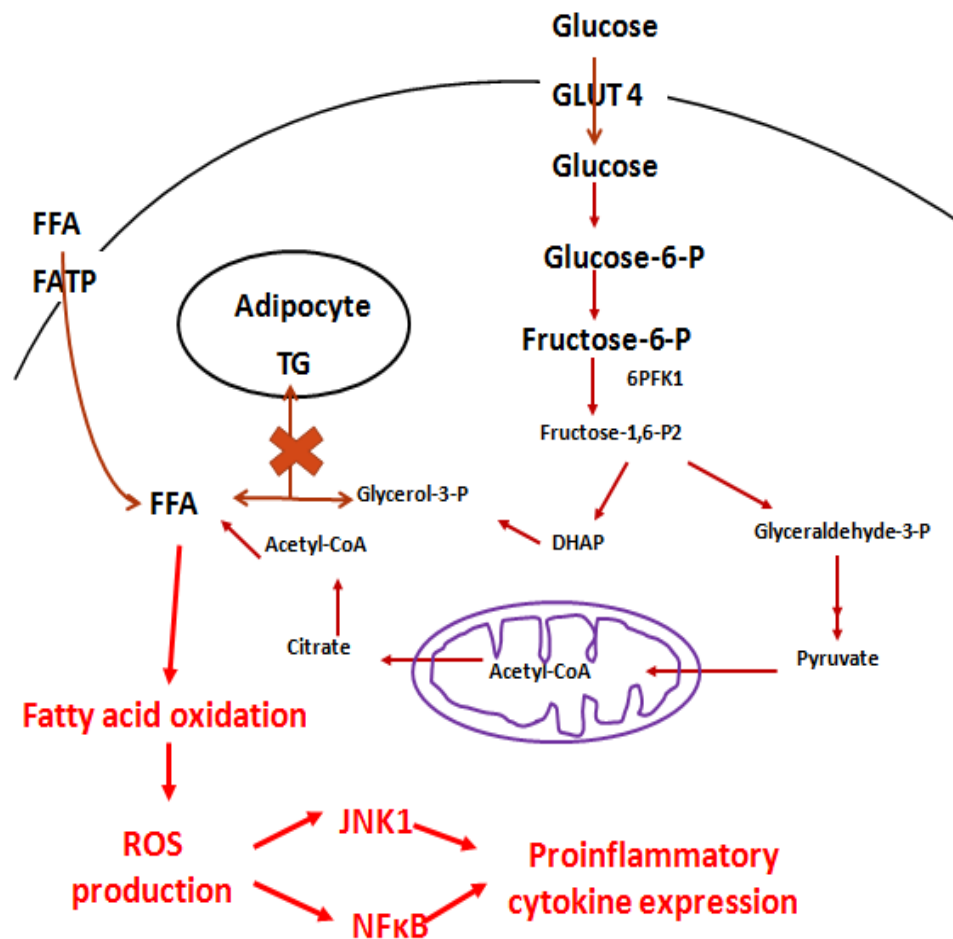


FIGURE 4. **Absence of PFKFB3/iPFK2 can lead to fatty acid oxidation.** Modified based on Guo, et al. (7).

According to Huo et al., after palmitate supplementation, iPFK2-KD adipocytes produced much more ROS compared to iPFK2-Ctrl, which indicating elevated amount of oxidative stress. In animal study, iPFK2-KD mice has significantly lower levels of adipose tissue F2,6BP and less lipid accumulation and increased oxidative stress, which lead to increase in JNK and NFκB p65 phosphorylation, increase in pro-inflammatory

cytokines expression such as TNF α and IL-6 and decrease in insulin signaling, compared to wild-type mice. Moreover, iPFK2-KD cell also had significantly lower expression of Akt phosphorylation which suggesting impaired in adipocyte function (6), as summarized in TABLE 1.

TABLE 1. **Effects of iPFK2-Ctrl and iPFK2-KD cells.** iPFK2-Ctrl is used as the control for iPFK2-KD. Data summarized from Huo et al. (6).

	TNF α	IL-6	Adiponectin	p-JNK/JNK	p-NF κ B/NF κ B
iPFK-Ctrl	-	-	-	-	-
iPFK2-KD	↑	↑	↓	↑	↑

After 1hr incubation with 100ng/mL LPS

Overnutrition causes excessive fatty acids oxidation in adipose tissue, thereby resulting in an increase in inflammatory response. The presence of PFKFB3/iPFK2 is expected to increase the production of glycerol-3-phosphate and FFA by glycolysis and pyruvate oxidation pathway. This appears to channel FFA to triglyceride synthesis, which in turns reduces the fatty acid oxidation-associated production of ROS. Ultimately, a decrease in ROS production protects against overnutrition-induced

activation of inflammatory signaling pathways of JNK and NF κ B, and suppresses the expression of proinflammatory cytokines (7).

2.4 Leucine

Leucine is one of the three branched-chain amino acids (valine, leucine and isoleucine) (33). Leucine is an essential amino acid that cannot be synthesized by humans and can only be obtained from diet. It is the most abundant amino acid found in human diet (33); example of leucine sources are legumes, whole grains and animal products such as dairy and eggs. Leucine is an important amino acid because it provides substrate for gluconeogenesis and regulates oxidation use of glucose by stimulating glucose recycling via glucose-alanine cycle (33). Leucine is also a functional amino acid, because it is a potent activator of mTOR (8).

Insulin initiates signaling at the insulin receptor, leading to activation of IRS-1 and PI3K. PI3K produces PIP3, leading to full activation of Akt by PDK1 and PDK2. Akt then phosphorylates TSC1/2 complex, which inactivates TSC2 GAP activity for GTP-bound Rheb, which is a potent activator of mTOR. Leucine, from dietary sources, also activates mTOR. mTOR is important for translation, cell size, cell growth and protein synthesis. However, activation of mTOR leads to activation of p70S6K to increase serine phosphorylation of IRS-1(34), as shown in FIGURE 5. By mechanism, dysregulation of mTOR will inhibit insulin signaling and insulin-stimulated glucose transport in muscles (35) and fat (36).

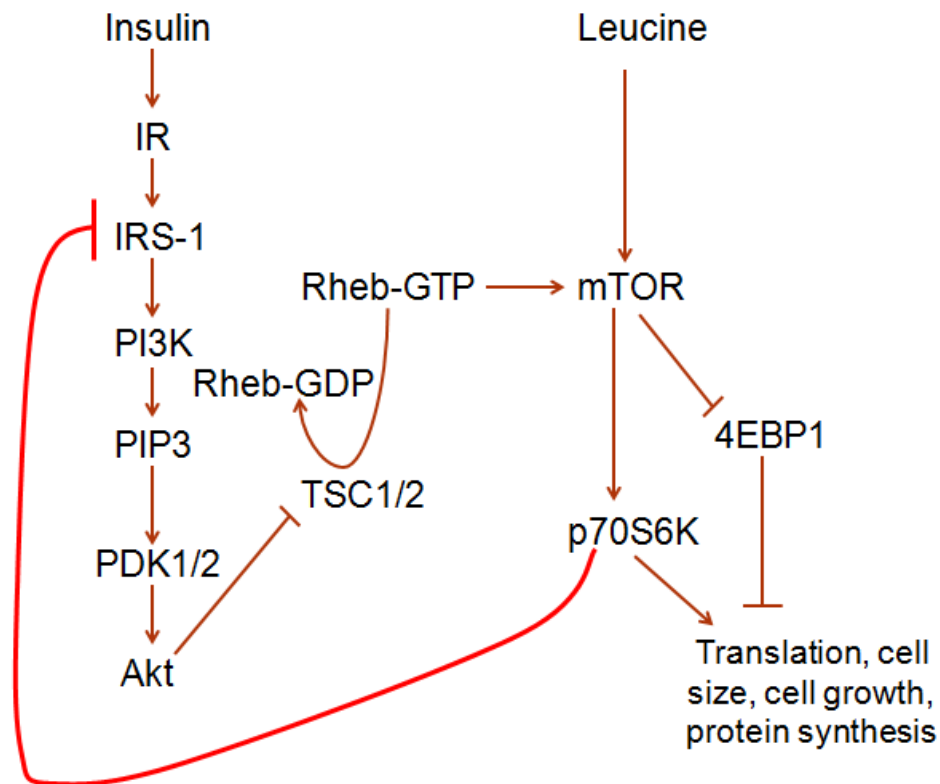


FIGURE 5. **mTOR activation.** Modified based on Corradetti, et al. (34).

To date, few studies have investigated the effects of leucine supplementation on glucose metabolism. Despite its effects by mechanism in inhibiting insulin signaling, dietary leucine supplementation has been shown to improve glucose tolerance, prevents hepatic steatosis, reduces obesity-induced adipose tissue inflammation and rescues insulin signaling in muscle, liver and fat (37). L-leucine supplementation improves strength performance in untrained male participants (38).

In HFD-fed mice, leucine administration had also found to reduce the weight gain and adiposity, and improves glucose metabolism by increasing insulin sensitivity by stimulating insulin secretion INS-1 cells via rapamycin-insensitive mechanism (39-40), decreasing plasma levels of glucagon and glucogenic amino acids and downregulate hepatic glucose-6-phosphatase (9). In healthy middle-aged adults, higher intake of BCAA (leucine, isoleucine and valine) has been associated with lower prevalence of overweight or obesity status (41). In protein-malnourished rats, leucine supplementation has been shown to increase glutamate dehydrogenase expression and restores glucose-induced insulin secretion (42). Leucine supplementation also shown to increase protein synthesis and to suppress protein catabolism in muscle, adipose and liver (43-44). In a study done by Hinault, et al., wortmannin, a pharmacologic PI3K inhibitor which blocks insulin-stimulated glucose transport and lipogenesis, and rapamycin, a potent inhibitor of mTOR, were used in the study. Upon addition of these two inhibitors, leucine still shown to allow insulin to activate Akt for insulin signaling. This study suggested that insulin sensitization of leucine may not mediated via the mTOR pathway (45), as shown in FIGURE 6.

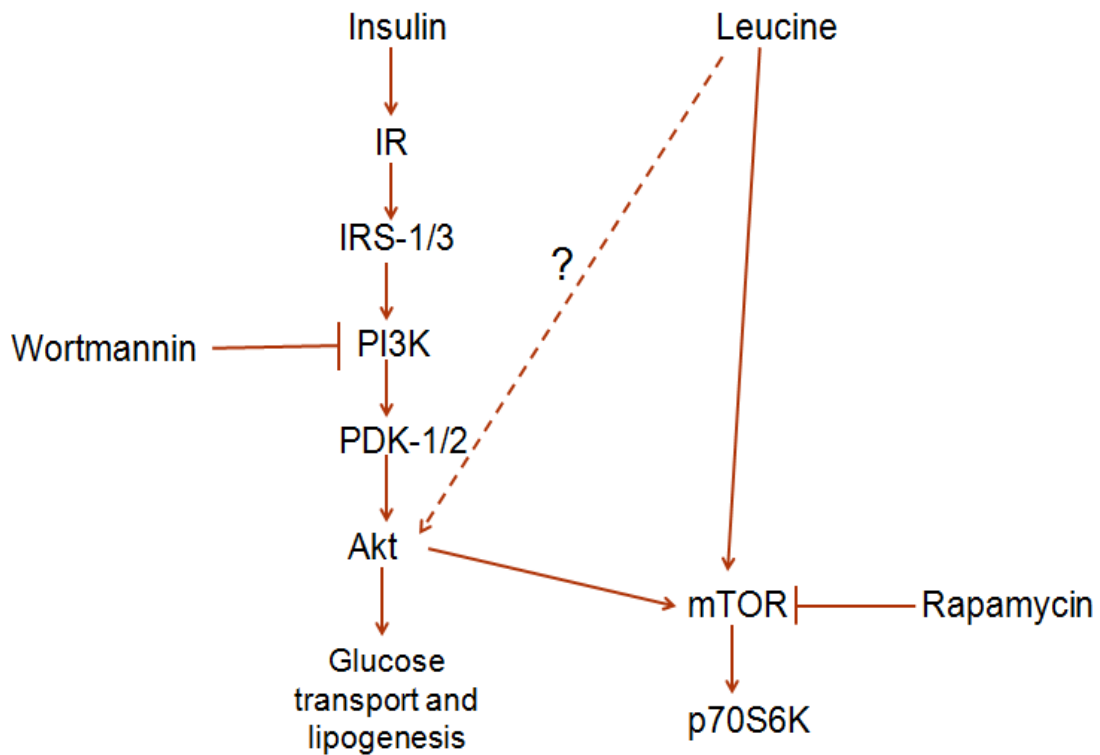


FIGURE 6. Effects of leucine on insulin sensitization may not mediated via the mTOR pathway. Modified based on Hinault, et al. (45).

2.5 n-3 PUFA metabolism, inflammatory responses and benefits

The n-3 PUFA, especially ALA, EPA, and DHA, has been studied extensively for its cardio protective effects in humans, cell cultures, epidemiology and animals (46). PUFA are dietary essential fatty acids, because it can only be obtained from diet in humans.

Humans and animals can synthesize SFA and MUFA, but not PUFA, because they do not have the enzyme needed (12- and 15-desaturase) to add cis double bond at the n-3 and n-6 position to synthesize ALA and LA (47). ALA derived from plants sources such as chia seed and flax seed (48), whereas EPA and DHA are from fish, fish oil supplements, and other seafood (48). Fish also do not synthesize n-3 PUFA, but the amount of n-3 PUFA in fish is high because they accumulate EPA and DHA by consuming plankton and algae (49).

Precursors for the synthesis of n-6 and n-3 PUFA are linoleic acid (18:2(n-6)) and α -linolenic acid (ALA; 18:3(n-3)) respectively. n-3 PUFA has first cis double bond positioned between the third and fourth carbon atoms from the methyl end of the fatty acids (47). Δ 6 desaturase is the enzyme needed to convert ALA to STA (18:4(n-3)). The metabolism of n-3 PUFA family ended with DHA (22:6(n-3)) as the end product, produced by Δ 5 desaturase enzyme to convert EPA (20:5(n-3)) to DHA (46). The n-6 and n-3 PUFAs metabolism is as shown in FIGURE 7.

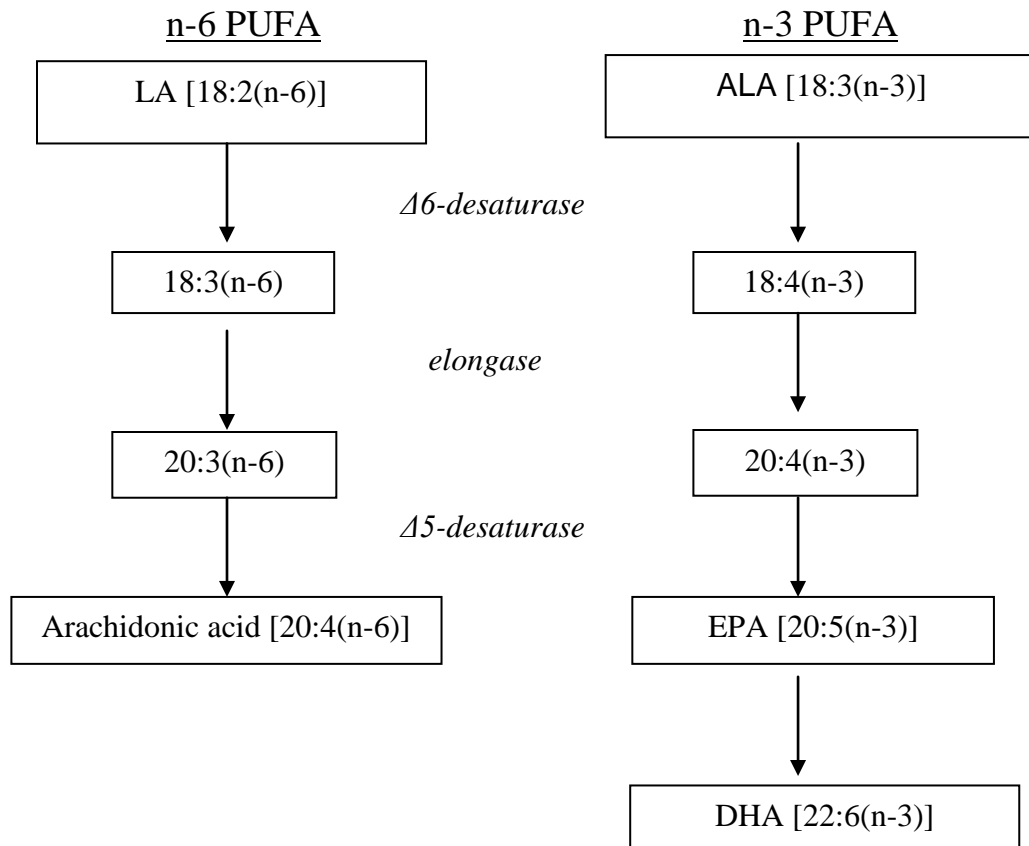


FIGURE 7. **Metabolism of PUFA.** Adapted and modified from Poudyal, et al. (46).

As mentioned before, human cannot synthesize PUFA; however, human have desaturase enzymes that can be used to elongate and desaturate PUFA. ER of the liver is the primary site for this metabolism (50), although it does take place in other tissues. LA and ALA compete for the enzyme for further metabolism, because both sharing common metabolic pathways (51).

Historically, human consumes almost the same ratio of n-6 PUFAs: n-3 PUFAs (52). Nowadays, the intake of LA has been increased dramatically due to the usage of LA-based vegetable oil such as soybean, sunflower and safflower oils (52) and limited consumption of sea foods in the U.S. population with combined intake of DHA and EPA is estimated at only 100 mg/d (53-54), with estimated ratio of at least 10:1 for n-6: n-3 PUFAs (55). It has been suggested that decreasing in ratio n-6: n-3 PUFAs may decrease the risk factors of metabolic syndrome, although the optimal ratio has not been recommended (56).

Canola oil, which is ALA-based vegetable oil, has been introduced in the last two decades, helps to increase the n-3 PUFA intake with average consumption of 1.3 g ALA/day (53-54). However, conversion of ALA to EPA and/or DHA is not very efficient, as described in the next paragraph.

An excessive consumption of LA would then delay the synthesis of EPA and DHA. According to the research, even without competing of the enzyme, the synthesis of EPA and DHA from ALA is quite inefficient because they are rapidly oxidized. Hence, EPA and DHA supplementation is more efficient and effective compared to ALA supplementation (50).

Extensive research had been done in the benefits of n-3 PUFA. DHA is required for visual and cognitive development of infants (57-59). The anti-inflammatory effects of n3-PUFA have improved many clinical symptoms such as in juvenile idiopathic arthritis patients (60). n-3 PUFA is also known for its anti-obesity factors; mice fed with high concentration of DHA and EPA, reduced its fat deposition and weight (61). It has

also been widely accepted that DHA and EPA prevents the development of heart disease by lowering triglyceride and increasing HDL-cholesterol levels (62-64). Some studies even suggested that DHA supplementation may be beneficial in protecting people from Alzheimer disease and other types of dementia (65-67).

However, the effects of n-3 PUFA in adipocytes, particularly in 3T3-L1 is still limited. From one of the studies of the effects of fatty acids in adipocytes 3T3-L1, palmitate (saturated fatty acids) had shown to increase inflammatory responses, such as TNF α , NF κ B, IL-6, ROS, JNK, and to decrease anti-inflammatory cytokines (IL-10) (13). On the other hand, DHA (n-3 PUFA) had been shown to suppress inflammatory responses (NF κ B and increase IL-10) and had no effect on TNF α (13), as summarized in TABLE 2.

TABLE 2. Effects of fatty acids in adipocytes 3T3-L1. Summarized from Ajuwon, et al. (13)

	ROS	JNK
Palmitate	↑	↑

	TNF α	NF- κ B	IL-6	IL-10
Palmitate	↑	↑	↑	↓
DHA	-	↓	n/a	↑

3. MATERIALS AND METHODS

The hypothesis of this study is that PFKFB3/iPFK2 is involved in the effects of leucine and/or n-3 PUFA on suppression of overnutrition-induced insulin resistance and inflammatory response in adipocyte.

The objectives of this study are: 1. Determine the extent to which PFKFB3/iPFK2 participates in the insulin-sensitizing effect of leucine in adipocyte; 2. Determine the extent to which PFKFB3/iPFK2 participates in the anti-inflammatory effect of n-3 PUFA in adipocyte.

3.1 Materials

These are the list of materials used in this study. Dexamethasone, insulin from porcine, 3-isobutyl-1-methylxanthine (IBMX), Dulbecco's Modified Eagle's Medium (DMEM), Trypsin EDTA, L-alanine, L-leucine, puromycin dihydrochloride from *Streptomyces alboniger*, and Penicillin-Streptomycin were purchased from Sigma (St. Louis, MO). Palmitoleic acid and linoleic acid were bought from Prep, Inc (MN). D-glucose, bovine albumin (BSA), DL-Dithiothreitol (DTT) were purchased from Amresco (Solon, OH). Fetal bovine serum (FBS) was from PAA Laboratories, Inc (Dartmouth, MA). Immobilon western chemiluminescent HRP substrate was ordered from Millipore Corp. (Billerica, MA). Antibodies for Akt 1/2/3 (H-136), p-Akt 1/2/3 (Ser473) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). TEMED (1,2-di(dimethylamino)ethane) was obtained from EMD chemicals Inc (Gibbstown, NJ).

ImmunoPure Antibody goat anti-rabbit IgG (H+L) horseradish peroxidase, Micro BCA Protein Assay Kit, were from Thermo Scientific (Rockford, IL). Prestained protein marker, broad range was ordered from BioLabs, Inc. RNA STAT-60 was purchased from Tel-Test, Inc (Friendswood, TX). Reverse transcription system was from Promega (Madison, WI). Water used was nanopure grade.

3.2 Cell culture

Stable PFKFB3/iPFK2-knockdown (iPFK2-KD) 3T3-L1 adipocytes and control (iPFK2-Ctrl) 3T3-L1 adipocytes, as well as wild-type 3T3-L1 adipocytes, were used in this study. Cell lines used in this study were previously generated by the Lab of Dr. Wu. iPFK2-Ctrl and iPFK2-KD cells were obtained by transfected predifferentiated 3T3-L1 cells with a plasmid containing shRNA vector (iPFK2-Ctrl) and shRNA against mouse PFKFB3 (iPFK2-KD) with Lipofectamine 2000 Transfection Reagent following manufacturer's protocol, respectively (6). To verify iPFK2-KD cells, cells were analyzed with Western blot and real-time RT-PCR for iPFK2 and PFKFB3 expressions, with iPFK2-Ctrl as control (6). The verification was performed ahead of this study.

Cells were maintained in high glucose DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin, and kept under humidified atmospheres at 37°C and 5% CO₂. Medium were replaced every other day until the cells reached 100% confluence. 5 µg/mL puromycin were added to iPFK2-KD and iPFK2-Ctrl cells to maintain the established stable cell lines (6).

To differentiate the cells, at 2 days of post-confluence, 3T3-L1 cells were treated with high glucose DMEM containing 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, 1 µg/ml insulin, 1 µM dexamethasone, and 0.5 mM IBMX for 48 hours. Then, fresh high glucose DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin and 1 µg/ml insulin were added for 2 days after the induction. Thereafter, the medium was changed to insulin-free medium every 2 days for additional 6 days. After cells are fully differentiated, treatments were added to the cells (6).

3.3 Treatments for leucine and n-3 PUFA on PFKFB3/iPFK2 gene expression

There were different treatments to determine the best dose for leucine and n-3 PUFA on adipocyte PFKFB3/iPFK2 gene expressions. Treatment for dose response with leucine on PFKFB3/iPFK2 gene expressions: 0 mM, 0.5 mM and 2.5 mM of alanine and leucine for 24 h. Alanine was used as a control for leucine. Treatment for dose response with DHA on PFKFB3/iPFK2 gene expression: 0 µM, 5 µM, 25 µM, 50 µM and 100 µM DHA for 24 h. To prepare leucine and alanine, PBS was used to dilute the leucine; thus, PBS was considered as 0mM leucine. For preparation of DHA, 10% BSA was used to dilute the DHA; thus BSA was considered as 0 µM DHA.

3.4 Treatment for leucine and n-3 PUFA on iPFK2-Ctrl and iPFK2-KD cells

After iPFK2-Ctrl and iPFK2-KD cells were fully differentiated, cells were divided into different sets for different treatments; 250 μ M palmitate was added for 48 h to induce the inflammation. Then, cells were treated with 0.5 mM leucine for 24 h before harvest. To determine the effect of n3-PUFA in different inflammatory responses, 50 μ M of LA or DHA were added to the cells for 24 h. LA was used as a control for DHA.

For insulin signaling responses, cells were treated with or without insulin (100 nM) for 30 min before harvest. After each treatment, cells were then harvested after washed twice with ice cold PBS. Cell lysis buffer was used to harvest protein samples; RNA STAT-60 (Tel-Test, Inc., Friendswood, TX) was used to harvest RNA samples, according to manufacturer's protocol. Samples were then analyzed with western blot and/or RT-PCR or real-time PCR depending on interested signaling and processed/analyzed as described later.

3.5 RNA isolation and RT-PCR

After cells were harvested, total RNA was extracted from cells with RNA STAT-60 following the manufacturer's protocol (Tel-Test, Inc., Friendswood, TX). RT-PCR reactions were performed using Reverse Transcription System Kit (Promega, Madison, WI), following manufacturer's protocol. The PCR was done using mastercycle egradient S (Eppendorf, Hamburg, Germany).

3.6 DNA agarose gel electrophoresis

RNA isolation and RT-PCR were conducted as previously described. DNA gel was made by boiling 1 g of Agarose with 100mL of 1X TAE buffer. Ethidium bromide was added to the agarose gel for visualization. cDNA samples were mixed with DNA loading buffer and loaded to DNA agarose gel and electrophoresed at 120V for 30-45min. Thereafter, gel was visualized with FluorChem under the UV light. Bands were measured using densitometry with ImageJ software (NIH, Bethesda, MD).

3.7 Real-Time PCR

After total RNA was extracted from cells, it is measured for RNA concentration with absorbance at 260nm. RNA was then diluted to 10ng/uL of total RNA. B-R 1-Step SYBR Green qRT-PCR Kit (Quanta Biosciences, Inc., Gaithersburg, MD) was used to run the samples for qPCR, following manufacturer's protocol. The RNA was analyzed for PFKFB3 in adipocyte cell samples. β -actin was used as the control.

3.8 Protein isolation and measurements

Cells were harvested with cell lysis buffer and left overnight at -80°C. Next, samples were centrifuged at 14,000 rpm at 4°C and supernatant, which contain the protein, was isolated for next step. Protein was then quantified with Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) following manufacturer's protocol. Protein concentration was measured with infinite M200 (Tecan, Switzerland) at 562nm absorbance. Protein was mixed with Laemmli's loading buffer and DTT to prevent

protein denaturation, boiled for 2-3min before loading to DNA gel for Western blot analysis.

3.9 Western blots

Samples were loaded to a gel with a broad-range prestained protein marker. Samples were then fractionated by electrophoresis using 10% polyacrylamide gels; the stacking gel was run at 60V for 45 min and resolving gel was run for 1.5 h at 110 V. The gels were transferred by wet blotting onto PVDF membranes at 25 V for 30 min. The membranes were then blocked with 5% skim milk in TBS for 2-3 hours with gentle shaking. Membranes were then incubated with a specific primary antibody against Akt 1/2/3 and phospho-Akt (Ser473) at 1:750 dilution at 4°C for overnight. The membranes were washed three times with TBS-T and incubated for 1.5 h with the secondary antibody conjugated with horseradish peroxidase (HRP) of antibody goat anti-rabbit at 1:7,500 dilution. Bands were developed using immobilon Western chemiluminescent HRP substrate (Millipore Corp., Billerica, MA) and were captured by CCD Camera (Cascade II:512, Photometrics, Tucson, AZ) using the WinView/32 software (Version 2.5, Princeton Instruments, Trento, NJ). Bands were measured using densitometry with ImageJ software (NIH, Bethesda, MD).

3.10 ROS measurements

Nitroblue tetrazolium (NBT) assay was used for ROS measurement. Before harvested, 0.2% NBT buffer was added to cells for 90 min, then cells were washed twice

with ice cold PBS. 0.5 mL 50% acetic acid was then added to each 3mL well before cells were scraped out. The absorbance was determined with infinite M200 (Tecan, Switzerland) at 560 nm (68).

3.11 Statistical analysis

Statistics for data presented as means \pm SE (standard error). Comparisons were made by two-tail t-test. Differences were considered significant if $P < 0.05$.

4. RESULTS AND DISCUSSIONS

4.1 Leucine

4.1.1 Wild-type 3T3-L1 on different doses of leucine treatment

To determine the best dosage for leucine treatment on PFKFB3 gene expression, fully differentiated wild-type 3T3-L1 cells were treated with 0.5mM and 2.5mM alanine and leucine for 24hr. Alanine was used as a control. Cells were then harvested and analyzed using RT-PCR to observe the PFKFB3 gene expression with 18s as the control. ImageJ software was used to quantify the densitometry of gene expressions. Result indicated that PFKFB3 expression induced more after 0.5mM leucine treatment (FIGURE 3). Means are shown (\pm SEM), n = 2. Two-tailed t-test was used to compare 0mM leucine with 0.5mM and 2.5mM leucine; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

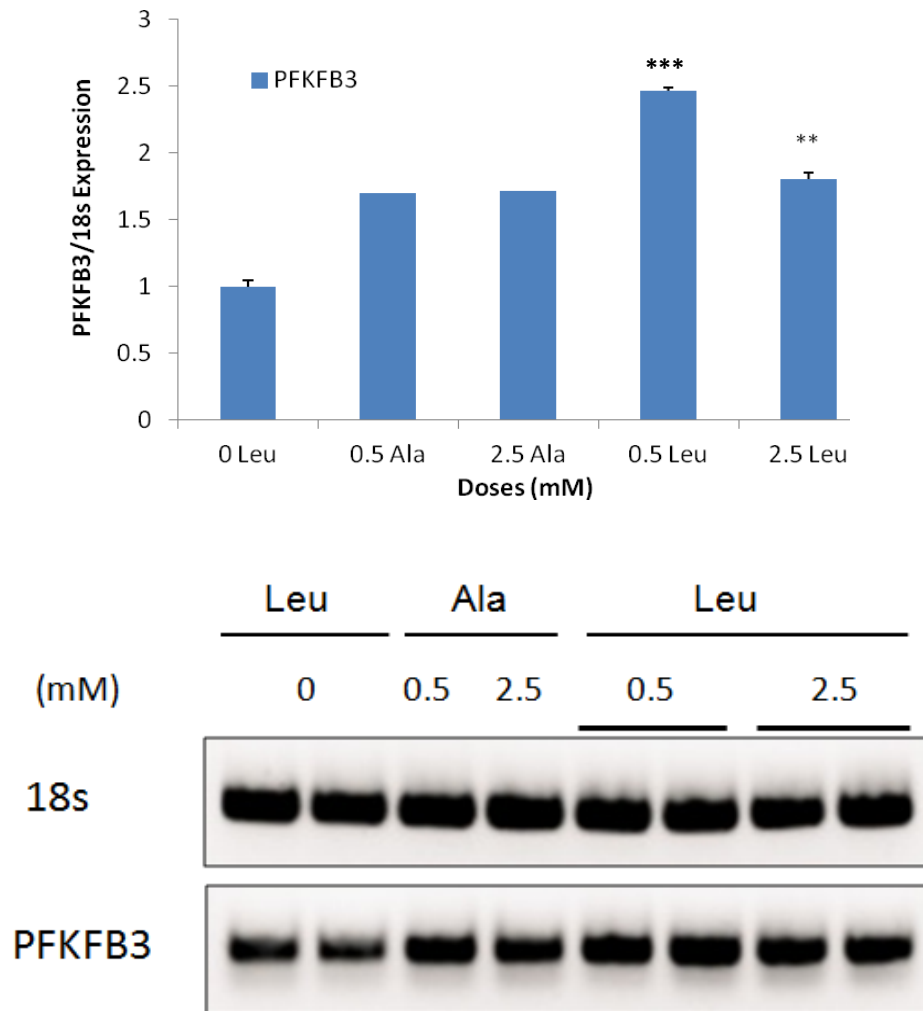


FIGURE 8. Wild-type 3T3-L1 on different leucine doses

4.1.2 Leucine treatment on insulin signaling of iPFK2-Ctrl and iPFK2-KD

iPFK2-Ctrl and iPFK2-KD cells were treated with 250 μ M palmitate for 48 hr to induce the inflammation; 0.5 mM leucine were added for 24 hr, with or without 100 nM insulin for 30 min before harvest. Protein were then harvested and analyzed with Western blot to observe the insulin signaling response of p-Akt/Akt. pAkt/Akt were used for measuring the insulin signaling in this experiment, because decreases in pAkt/Akt signaling might indicate decreases in IRS-1 activation, thus decrease in insulin signaling (16). ImageJ software was used to quantify Western blot data.

On iPFK2-Ctrl and iPFK2-KD cells, as expected, palmitate decreased insulin signaling; palmitate with insulin (Pal+) had lower p-Akt/Akt expressions compare to basal. When comparing Pal+ with Leu+, there was an obvious increase in p-Akt/Akt expressions compared to PBS+ and Pal+. The insulin-sensitizing effect of leucine was also shown in Pal/Leu+ treatment; Pal/Leu+ had stronger p-Akt/Akt expression compares to Pal+.

However, result of iPFK2-Ctrl and iPFK2-KD of leucine+ treatment indicated that insulin-sensitizing effect of leucine had been diminished in iPFK2-KD cells. Data suggested that leucine supplementation increases insulin sensitivity in iPFK2-Ctrl cells and suggesting that PFKFB3/iPFK2 is involved in the insulin-sensitizing effect of leucine treatment (FIGURE 9).

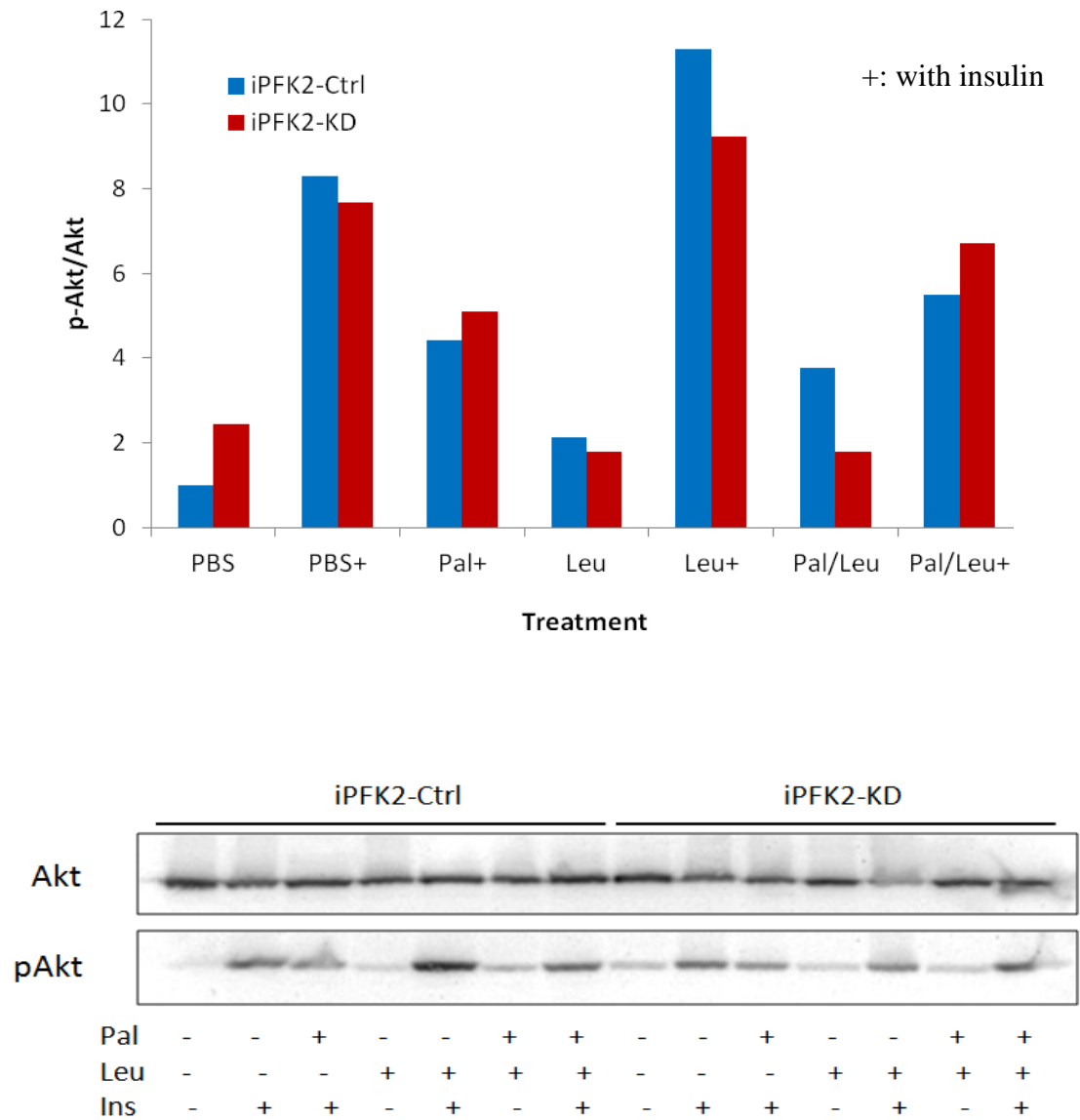


FIGURE 9. Effects of leucine on insulin signaling of iPFK2-Ctrl and iPFK2-KD cells.

4.1.3 Leucine treatment on cytokine expression of iPFK2-Ctrl and iPFK2-KD

After cells were fully differentiated, different treatments were added to the cells: palmitate was added to induce the inflammation, alanine was used as a control for leucine. Dose used for leucine was 0.5 mM for 24hr. RNA was then analyzed with real-time RT-PCR. Means are shown (\pm SEM), $n = 3$ of normalized iPFK2-KD to iPFK2-Ctrl obtained from relative abundance of cytokine expressions. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

In adiponectin expression, as expected, upon Pal/PBS treatment, it decreases adiponectin expression, compare to the basal (BSA/PBS). Pal/Leu supplementation was expected to recover or increase adiponectin expression compare to Pal/PBS. However, to our surprise, leucine supplementation appears to have limited effect on adiponectin expressions, with $^{\dagger} P$ value of 0.0263, $n = 3$. In both iPFK2-Ctrl and iPFK2-KD, statistical comparison of iPFK2-Ctrl and iPFK2-KD indicated that iPFK2-KD cells expressed significantly lower adiponectin. (FIGURE 10).

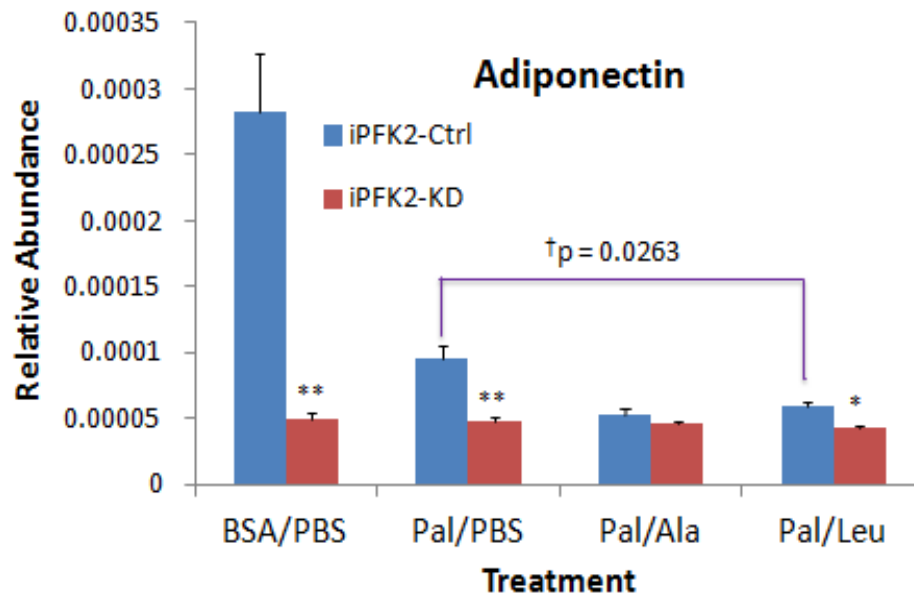


FIGURE 10. Effects of leucine on adiponectin expression of iPFK2-Ctrl and iPFK2-KD.

In TNF α expression, as expected, Pal/PBS had higher TNF α expression compared to basal (BSA/PBS). This effects, appear to be improved after alanine and leucine supplementations in both iPFK2-Ctrl and iPFK2-KD cells. To our surprise, Pal/Ala had more suppression on TNF α expression compare to Pal/Leu. However, statistically, Pal/PBS and Pal/Leu had no significant difference, with P-value of 0.2392 (FIGURE 11).

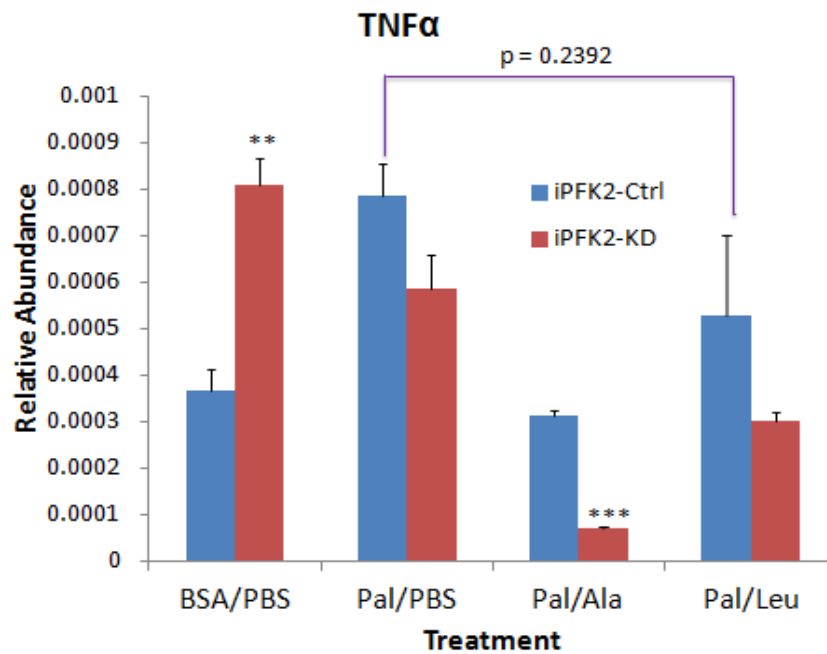


FIGURE 11. Effects of leucine on TNF α expression of iPFK2-Ctrl and iPFK2-KD cells.

These experiments suggest that leucine supplementation appears to have no effect on iPFK2-KD adiponectin expression and does not decrease proinflammatory cytokine expression.

4.1.4 Leucine treatment on ROS of iPFK2-Ctrl and iPFK2-KD

After cells were fully differentiated, different treatments were added to the cells: palmitate was added to induce the inflammation, alanine was used as a control for leucine. Dose used for leucine was 0.5 mM for 24 hr. Nitroblue tetrazolium (NBT) assay was used for ROS measurement. Means are shown (\pm SEM), n = 2 of normalized iPFK2-

KD to iPFK2-Ctrl obtained from ROS measurement. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Statistics indicated that when comparing iPFK2-Ctrl and iPFK2-KD cells under the same condition, ROS increased in iPFK2-KD cells; with *** $P < 0.001$ on BSA and Pal/Ala, ** $P < 0.01$ on BSA/Leu and Pal, and * $P < 0.05$ on BSA/Ala and Pal/Leu; supporting that with the presence of PFKFB3/iPFK2 genes, ROS production decreases. However, there were no significant changes on cells with or without leucine supplementation, suggesting that leucine supplementation appears to have limited effect on reducing ROS production (FIGURE 12).

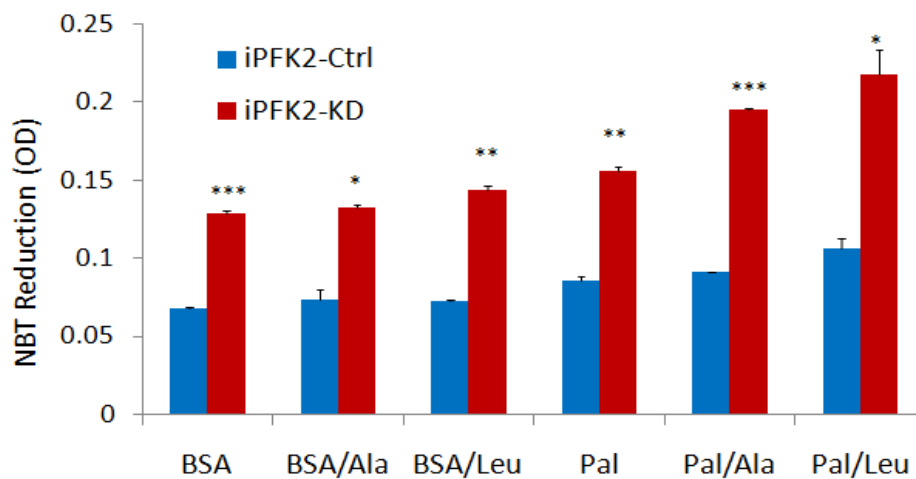


FIGURE 12. Effects of leucine on ROS of iPFK2-Ctrl and iPFK2-KD cells.

4.2 n-3 PUFA

4.2.1 Wild-type 3T3-L1 on different doses of n-3 PUFA treatment

Wild-type 3T3-L1 cells were treated with 5 μM , 25 μM , 50 μM and 100 μM for 24 hr. RNA was then harvested and analyzed using qPCR to observe the PFKFB3 gene expression with β -actin as the control. Means are shown (\pm SEM), $n = 3$. Two-tailed t-test was used to compare 5 μM DHA with 25 μM , 50 μM and 100 μM DHA; * $P < 0.05$.

There was a significant difference upon treatment with 25 μM and 100 μM , however, 100 μM DHA appears to decrease the PFKFB3 expression, which might be due to viability issues. Thus, PFKFB3 expression induced more upon 25 μM DHA treatment (FIGURE 13).

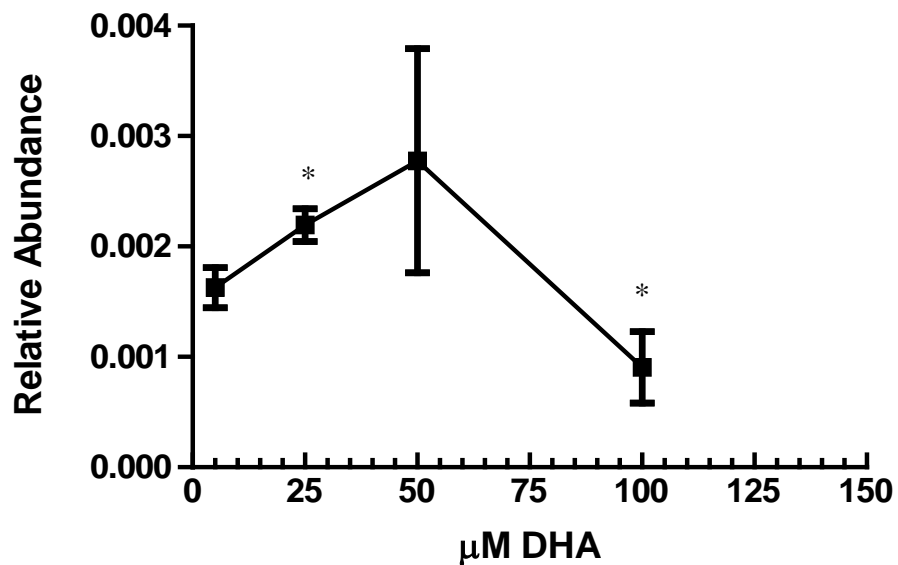


FIGURE 13. Wild-type 3T3-L1 on different DHA doses.

4.2.2 n-3 PUFA treatment on insulin signaling of iPFK2-Ctrl and iPFK2-KD

iPFK2-Ctrl and iPFK2-KD cells were treated with 0.5 mM DHA for 24 hr; with or without 100 nM insulin for 30 min before harvest. LA was used as control for DHA. Cells were then harvested and analyzed with western blot for insulin signaling response of p-Akt/Akt. ImageJ software was used to quantify the Western blot.

Results shown that under the same condition, iPFK2-KD cells had lower p-Akt/Akt expressions compared to iPFK2-Ctrl cells, indicating insulin sensitivity is decreased in iPFK2-KD cells. In both iPFK2-Ctrl and iPFK2-KD cells, DHA supplementation with insulin (DHA+) did not increase p-Akt/Akt expressions, suggesting that DHA supplementation appears to have limited effect on insulin sensitization (FIGURE 14).

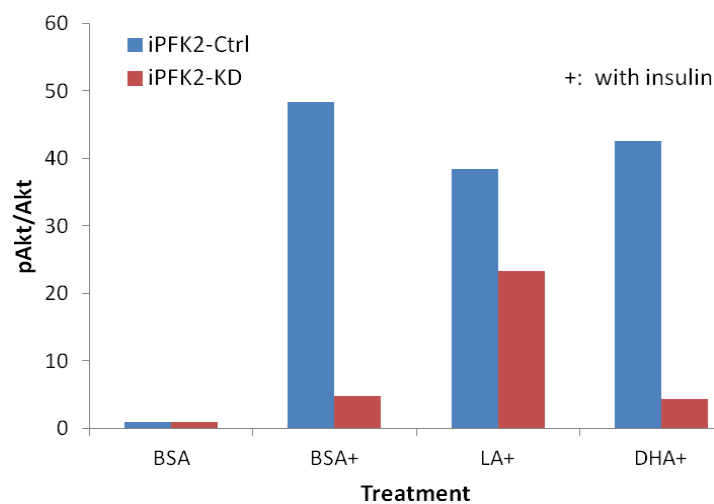
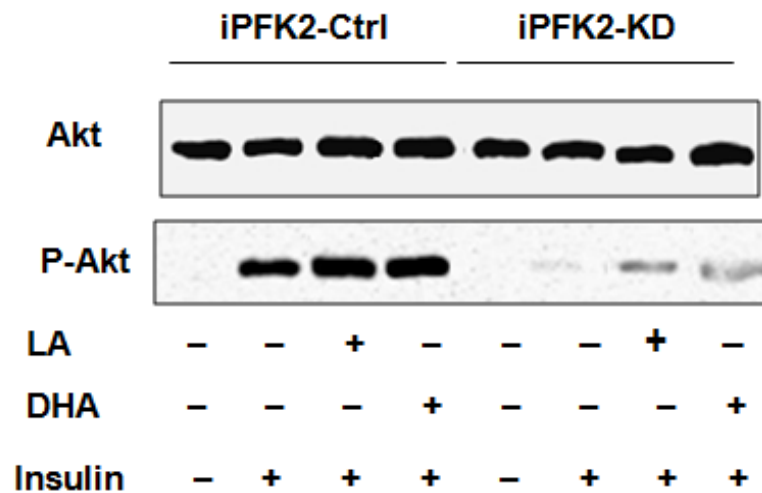


FIGURE 14. Effects of DHA on insulin signaling of iPFK2-Ctrl and iPFK2-KD cells.

FIGURE 14. **Continued.**

4.2.3 *n-3 PUFA treatment on cytokine expression of iPFK2-Ctrl and iPFK2-KD*

After cells were fully differentiated, different treatments were added to the cells: palmitate was added to induce the inflammation, LA was used as a control for DHA. Dose used for DHA was 50 μ M for 24 hr. RNA was then analyzed with RT-PCR. 18s was used as the control. ImageJ software was used to quantify RT-PCR results.

In iPFK2-Ctrl cells, LA and DHA supplementation appeared to have the same TNF α and IL-6 expression compared to Pal/PBS treatment. Moreover, in iPFK2-KD cells, TNF α and IL-6 expression were stronger after DHA supplementation (FIGURE 15). This data suggests that DHA supplementation appears not to reduce pro-inflammatory cytokine expression in iPFK2-KD cells.

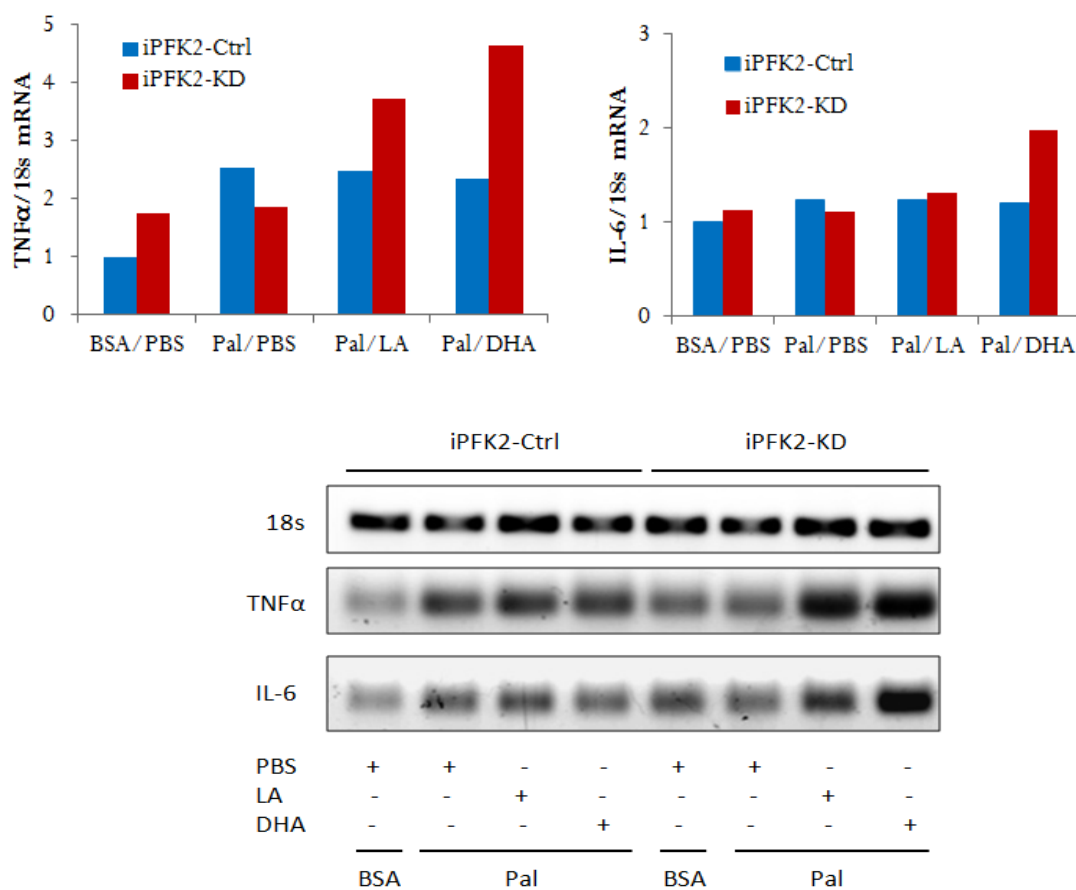


FIGURE 15. Effects of DHA on cytokine expression of iPFK2-Ctrl and iPFK2-KD cells.

4.2.4 n-3 PUFA treatment on ROS of iPFK2-Ctrl and iPFK2-KD

After cells were fully differentiated, different treatments were added to the cells: palmitate was added to induce the inflammation, LA was used as a control for DHA. Dose used for DHA was 50 μ M for 24 hr. Nitroblue tetrazolium (NBT) assay was used for ROS measurement. Means are shown (\pm SEM), $n = 2$ of normalized iPFK2-KD to iPFK2-Ctrl obtained from ROS measurement with ** $P < 0.01$.

Statistics indicated that comparing iPFK2-Ctrl and iPFK2-KD cells under the same condition, ROS increased in iPFK2-KD cells; with ** $P < 0.01$ on BSA, BSA/LA, Pal and Pal/LA supplementations; supporting that with the presence of PFKFB3/iPFK2 genes, ROS production decreased. However, there were no significant changes on cells with or without DHA supplementation, suggesting that DHA supplementation appears to have limited effect on reducing ROS production (FIGURE 16).

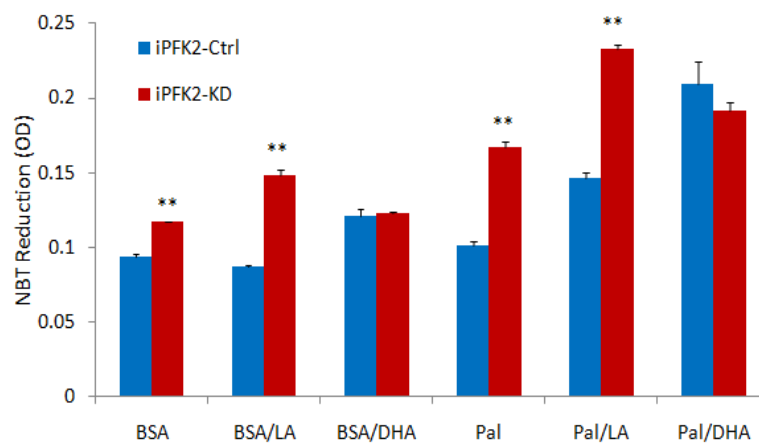


FIGURE 16. Effects of DHA on ROS of iPFK2-Ctrl and iPFK2-KD cells.

5. SUMMARY AND CONCLUSIONS

5.1 Summary

The results of this study suggest the PFKFB3/iPFK2 gene expression is induced more with 0.5mM leucine. For DHA supplementation, 25 μ M DHA is the best dose to stimulate PFKFB3/iPFK2 gene expression.

The results of leucine study suggest that PFKFB3/iPFK2 is involved in the insulin-sensitizing effect of leucine treatment. In this study, leucine supplementation on iPFK2-Ctrl cells appear to have insulin sensitizing effects through p-Akt/Akt signaling, but not on adiponectin expression. However, leucine supplementation appears to have limited effect on reducing proinflammatory nor increasing anti-inflammatory cytokine expressions and ROS production.

From the study, it has also been shown that DHA supplementations on iPFK2-Ctrl cells appear to have limited effects on insulin sensitizing, suppressing pro-inflammatory and reducing ROS production. In iPFK2-KD cells, supplementation of DHA appears not to reduce in ROS production. Moreover, proinflammatory cytokine expression is stronger in iPFK2-KD treated cells.

5.2 Conclusions

The results of this study support the hypothesis that PFKFB3/iPFK2 is critically involved in insulin-sensitizing effects of leucine. This role of PFKFB3/iPFK2, however,

appears to be independent of anti-inflammatory responses. Given this, it is likely that PFKFB3/iPFK2 only account, in part, for the beneficial effects of leucine.

n-3 PUFA stimulate PFKFB3/iPFK2 activity in wild-type adipocytes. However, PUFA do not exhibit anti-inflammatory and insulin-sensitizing effects in controls. In contrast, n3-PUFA exhibit proinflammatory effects in iPFK2-KD cells.

Taken together, PFKFB3/iPFK2 is involved, at least in part, in the effects of insulin sensitization of leucine and appears to protect adipocytes from inflammatory responses, which could be exacerbated by n-3 PUFA when PFKFB3/iPFK2 is disrupted.

5.3 Future experiments

Suggestions for future experiments following this study are:

1. Using other antibody such as upstream regulators of Akt/pAkt to study the insulin sensitization effects of leucine
2. Add a substrate to the iPFK2-KD cells to reverse the effects of PFKFB3-knockdown, to confirm the role of iPFK2-Ctrl cells on protecting adipocytes from inflammatory responses
3. Design prevention studies on the involvement of PFKFB3/iPFK2 in the effects of leucine and n-3 PUFA, rather than treatment experiments, which was already presented in this thesis

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