NUTRIENT REGULATION OF PFKFB3/iPFK2 AND ITS ROLE IN REGULATING DIET-INDUCED INFLAMMATION IN INTESTINAL EPITHELIAL CELLS

A Dissertation

by

RACHEL ERIN BOTCHLETT

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Chair of Committee,	Chaodong Wu
Committee Members,	Joseph M. Awika
	Steven Riechman
	Rosemary Walzem
Head of Department,	Boon Chew

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ABSTRACT

The gene PFKFB3 encodes for inducible 6-phosphofructo-2-kinase (iPFK2), an important regulatory enzyme of glycolysis. It is shown that PFKFB3/iPFK2 links metabolic and inflammatory pathways in adipose tissue; however, whether it functions in the same manner within small intestine, where nutrients are assimilated and first interact with the body, is unknown. Therefore, the present study firstly investigated how diet, macronutrients, e.g. glucose and palmitate, and bacterial metabolites influence PFKFB3/iPFK2 expression, and secondly determined how altered gene expression relates to inflammatory responses in small intestinal epithelial cells (IECs).

HFD feeding and *in vitro* palmitate treatment were associated with reduced PFKFB3/iPFK2 but increased proinflammatory responses. LFD feeding and glucose treatment showed the opposite result. *In vitro* overexpression of PFKFB3/iPFK2 lead to reduced proinflammatory responses while inhibition of PFKFB3/iPFK2 was associated with increased inflammatory markers. Treatment with the bacterial metabolite indole stimulated PFKFB3/iPFK2 and reduced the generation of inflammation.

Together these findings indicate that macronutrients differentially regulate PFKFB3/iPFK2 expression in IECs, where carbohydrates stimulate PFKFB3/iPFK2 and saturated fats contribute to proinflammatory mechanisms. Further, results confirm an anti-inflammatory ability of PFKFB3/iPFK2 within IECs and suggest an additional anti-inflammatory mechanism of action of indole in regulating inflammation through PFKFB3/iPFK2.

DEDICATION

For my husband, Jason, without whom I would not be where I am today. Your love, guidance, support, and inspiration made it possible for me to pursue a PhD and complete this work. I am forever grateful for all you have sacrificed for me to accomplish this goal. I love you.

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NOMENCLATURE

3PO	3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one
BSA	Bovine Serum Albumin
DMEM	Dulbecco's Modified Eagle's Medium
FE	Feeding Efficiency
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GTT	Glucose Tolerance Test
HFD	High Fat Diet
HG	High Glucose
IMDM	Iscove's Modified Dulbecco's medium
ITT	Insulin Tolerance Test
IEC	Intestinal Epithelial Cell
IL-6	Interleukin-6
iPFK2	Inducible 6-phosphofructo-2-kinase
IMDM	Iscove's Modified Dulbecco's medium
JNK	c-Jun N-terminal kinase
LFD	Low Fat Diet
LG	Low Glucose
LPS	Lipopolysaccharide
NFκB	Nuclear factor kappa beta

PBS	Phosphate Buffered Saline
PGC1α	Peroxisome Proliferator Activated Receptor Gamma Co-activator 1 Alpha
pPARγ	Peroxisome Proliferator Activated Receptor Gamma
PFKFB3	6-phosphofructo-2-kinase/ fructose-2,6-bisphophatase 3
PI3K	Phosphoinositol-3-kinase
pAkt	Phosphorylated Akt
pNFκβ	Phosphorylated NFκβ
pJNK	Phosphorylated JNK
ROS	Reactive Oxygen Species
SCFA	Short Chain Fatty Acid
SI	Small Intestine
TLR4	Toll-like Receptor 4
TNFα	Tumor Necrosis Factor alpha
WAT	White Adipose Tissue

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CHAPTER I

INTRODUCTION

Much research has investigated the role of the adipose tissue and liver in regulating adiposity, glucose homeostasis, and the generation of inflammation in obesity; however, research is only starting to focus on the role of the small intestine. Metabolic and inflammatory signaling within the small intestinal epithelial cells (IECs) in particular is largely unknown but especially important to study as they are the primary cells for nutrient absorption and where nutrient-host cell interactions first occur. For example, adverse effects from overnutrition for instance are thought to initially occur in the intestine and contribute to systemic effects secondarily. Therefore, this proposal is aimed at investigating the mechanism(s) as to how HFD-induced obesity alters metabolic gene expression and contributes to the generation of inflammatory responses in IECs.

PFKFB3 is an important metabolic gene to regulate glycolysis in multiple tissues and cell types including adipose tissue, small intestine, and macrophages. It has been previously shown that PFKFB3 expression in the whole small intestine tissue is increased in response to HFD feeding; however, no one has examined PFKFB3 expression in response to the same condition in IECs. Understanding how IEC PFKFB3 expression changes in disorders such as obesity and associated metabolic diseases is necessary to determine how impairments in this gene negatively affect nutrient absorption or metabolic signaling and thus, may contribute to inflammation. Thus, the **central hypothesis** of this

project is that PFKFB3 serves as a link between diet and metabolic and inflammatory responses in IECs. The **overall goal** of this research is to provide evidence for the importance of PFKFB3 in IECs, a topic that is not well investigated. The **significance** of this work is that it demonstrates a novel role of PFKFB3 in IECs and provides experimental evidence for reducing HFD-induced intestine inflammation through regulating PFKFB3 expression.

CHAPTER II

LITERATURE REVIEW

OBESITY AND METABOLIC DISORDERS

Obesity, a health condition characterized by excess body fat, has become a major health issue in the United States especially, as over one-third of American adults are now classified as obese (1). This number has greatly increased over the past 20-30 years (Figure 1; 2) and is expected to continue to rise over the next several decades (3).

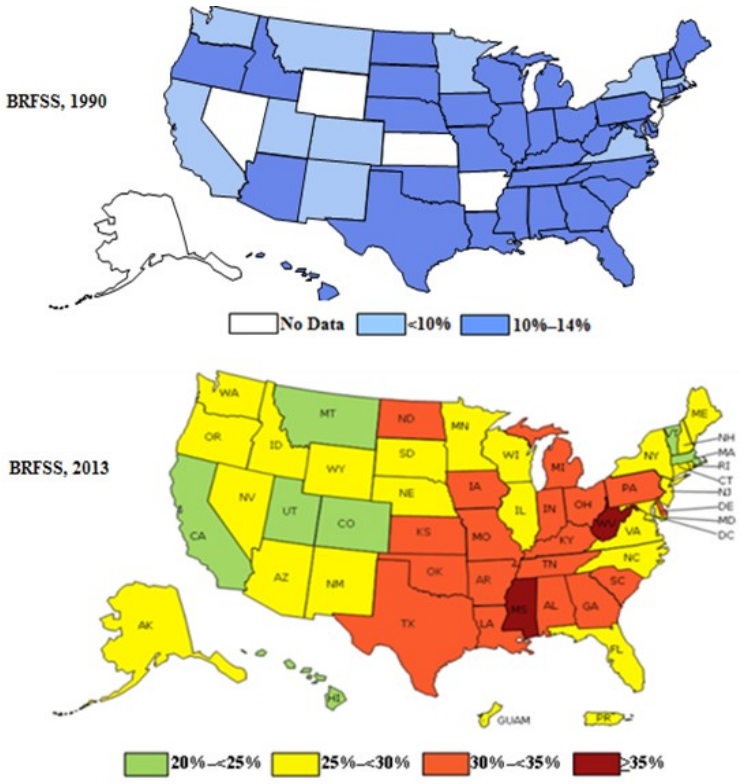

Figure 1. Obesity trends among American adults [2].

While several factors including smoking (4), genetic mutations (5,6), and endocrine disorders (7) are known to increase the risk of developing obesity, its onset is most commonly caused by a combination of inactive lifestyle and unhealthy eating behaviors such as overnutrition. Overnutrition with a high-fat diet (HFD), which contributes >35% calories from fat, is a particularly common cause of obesity as fat consumption directly increases both subcutaneous and visceral white adipose tissue (WAT; 8,9), the primary fat storage organ in the body. In addition, HFD feeding in many species, including humans, is associated with morphological changes in the intestine, including an increased number and length of villi (Figure 2; 10,11), which ultimately increases the efficiency of fat absorption and assimilation into the body (12,13). The combination of enlarged WAT and increased intestinal fat absorption culminates in HFD-induced obesity, which is correlated with abnormal lipid metabolism and dyslipidemia (14), increased oxidative stress and inflammation (15-17), increased intestinal permeability (18), and a decrease in systemic insulin sensitivity (19). Obesity is considered a major risk factor for the development of metabolic disorders including atherosclerosis, heart disease, some forms of cancer, hypertension, metabolic syndrome and type 2 diabetes mellitus (T2DM; 20-23). In fact, it seems that obesity-induced inflammation, in both animal models of obesity as well as obese human patients, may be the central causal factor in the development of insulin resistance and T2DM (24,25).

INSULIN RESISTANCE/ TYPE 2 DIABETES MELLITUS

Pathology

T2DM is the most common form of diabetes mellitus, accounting for almost 95% of diabetic cases, and is characterized by insulin resistance. As opposed to Type 1 diabetes mellitus, where insufficient insulin is produced by pancreatic beta cells, in T2DM insulin is produced at physiological levels but tissues are unresponsive or less responsive to the insulin signal. In normal insulin signaling, insulin binds to the insulin receptor (IR) and initiates a secondary signaling cascade which includes the phosphorylation of the insulin receptor substrate substrates 1 and 2 (IRS1/2), phosphatidylinositol-3-kinase (PI3K), and Akt, and the subsequent translocation of the glucose transporter (commonly GLUT1-4)

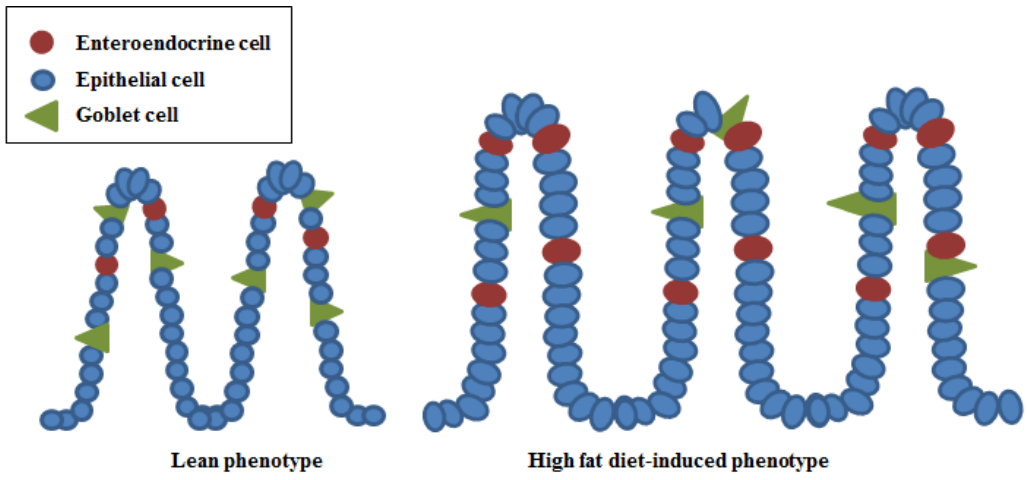

Figure 2. HFD-induced changes in morphology of small intestinal microvilli (Modified from 11).

from the cytosol to the cell membrane to facilitate glucose entry into the cell. However, with insulin resistance/ T2DM insulin is partially recognized by the IR and/or secondary

signaling pathways are ineffectively activated resulting in glucose accumulation in the bloodstream (i.e. hyperglycemia). In addition, in insulin resistant individuals, normal ability to suppress hepatic glucose production in response to elevations in circulating insulin levels is reduced. Continuous hepatic glucose output, when excessive, contributes to the development of hyperglycemia. Hyperglycemia can contribute to the onset of many health conditions ranging from poor circulation and blood vessel damage to heart disease and stroke (26,27). Fortunately, short term insulin resistance, or pre-diabetes, can be reversed through moderate exercise and calorie-appropriate diets (23). Once T2DM has been diagnosed however, it is extremely difficult to reverse the disorder and patients must continuously monitor sugar intake, blood glucose concentrations, and consume anti-diabetic medication, such as metformin (28), to regulate blood glucose.

Systemic effects

T2DM, if not monitored, can be very dangerous. Abnormal insulin signaling and thus, insufficient glucose uptake can be disastrous to many cell types and tissues that require glucose as a primary source of energy, for example the brain, red blood cells and kidneys. Further, insulin resistance has been shown to impair normal metabolic signaling in primary tissues involved in glucose homeostasis. For example, reduced insulin signaling in the liver leads to unregulated glucose production because insulin is required to halt hepatic glucose release when blood glucose levels are high. In skeletal muscle, the major organ for glucose utilization, impaired insulin signaling leads to reduced glycogen synthesis (29) which can greatly contribute to hyperglycemia. Further, insulin resistance

in adipose tissue causes the failure of insulin-mediated suppression of lipolysis (30,31) resulting in increased free fatty acid (FFA) release, which are damaging at high concentrations. T2DM, especially when paired with obesity, can also severely interfere with normal nutrient-sensing mechanisms (32) and lipid metabolism (33) in the small intestine, which leads to reduced nutrient absorption and an overall imbalance in metabolic homeostasis. All of these impairments in turn exacerbate T2DM, and can lead to a vicious cycle of insulin resistance and metabolic dysfunction.

Causes/underlying mechanisms

T2DM is a complex disorder that can be caused by a multitude of factors including genetic mutations (34) or loss/inhibition of the IR (35,36). However, because T2DM is statistically linked to obesity, much research focuses on the underlying mechanisms of overnutrition-induced insulin resistance. Long-term HFD feeding experiments are especially useful as they allow researchers to study the onset of obesity and insulin resistance concomitantly. It is well known that HFD directly contributes to obesity and insulin resistance primarily through adipocyte hypertrophy. When adipocytes become overly enlarged they display abnormal lipid metabolism, which results in 2 major consequences: impaired fatty acid synthesis and oxidation (37), and increased FFA release from adipocytes. Excess FFAs enter the bloodstream and accumulate in various tissues causing steatosis (38) and systemic insulin resistance. For example, increased FFAs impair the insulin signal in muscle (29), and provide increased substrate for hepatic gluconeogenesis, which results in increased glucose production (39) and an exacerbated

hyperglycemic phenotype. Further, excess FFAs are toxic to pancreatic beta cells and can cause reduced insulin production (40). HFD can also directly impair lipid metabolism in other tissues, including the liver and kidney. Specifically, it leads to reduced lipolysis through inhibition of 5' AMP-activated protein kinase (AMPK; 41,42) and increased lipid accumulation via increased expression of fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC; 43,44), all of which can intensify systemic insulin resistance.

Overnutrition-induced interference to insulin signaling

In addition to its effect in adipocytes, HFD is also known to interfere with several sites in the insulin signaling cascade in non-adipose tissues (Figure 3). For example, HFD reduces IR and IRS1/2 expression and/or phosphorylation (45-47), impairs the phosphorylation of PI3K (48,49) and Akt (46,50,51), severely diminishes GLUT4 expression (52,53), and alters GLUT2/4 translocation to the cell membrane (45,51). Many studies are now suggesting that inflammation generated from the diet-induced obese (DIO) phenotype is the key contributor to altered insulin signaling and systemic insulin resistance/T2DM. Therefore, the generation of inflammatory cytokines within the context of overnutrition, and their contribution to T2DM, will be discussed in detail in the following section.

INFLAMMATION

Overnutrition-induced inflammation

Pro-inflammatory cytokines are proteins naturally produced throughout the body in response to harmful stimuli such as pathogens or physical stress. They serve to regulate cell signaling pathways and initiate the innate immune response in almost all cell types. For example, during injury cells secrete inflammatory cytokines to recruit macrophages and lymphocytes to repair damaged areas. An acute or short-term inflammatory response is necessary to maintain cell viability and function and fight off infection; however, chronic generation of inflammatory cytokines leads to an inflamed phenotype and can result in tissue damage (54) and impaired cell signaling (55). With the increasing prevalence of obesity and metabolic diseases over the past several decades there is now a better understanding of overnutrition-induced inflammation. It is known that long-term overnutrition/high energy intake leads to increased fat/lipid synthesis and in turn, abnormally enlarged adipocytes and impaired lipid metabolism. Aberrant adipocytes secrete inflammatory cytokines including tumor necrosis factor alpha (TNFα) and interleukin-6 (IL-6), as well as stimulate expression of the transcription factor nuclear factor kappa beta (NFκB; 56; Figure 4), all of which recruit macrophages into adipose tissue. However, with overnutrition, such as high carbohydrate intake or a chronic HFD, macrophages cannot counteract the continuous production of cytokines by damaged adipocytes and in turn, they themselves generate inflammatory cytokines such as interleukin 1-β (IL1-β; 57; Figure 4). The accumulation of damaged adipocytes, macrophages, and local inflammation ultimately leads to the generation of inflammation

in distal tissues. For this reason a defining characteristic of obesity is chronic, low-grade systemic inflammation.

Given that obesity is highly associated with T2DM, recent research has focused on the underlying mechanisms of how obesity-induced inflammation contributes to insulin resistance. In fact, the term "metainflammation" is now being used to describe the idea that inflammation is the primary causal factor in many metabolic diseases (58,59). Inflammation has even been identified as a key component in some forms of cancer (60) and in the development of atherosclerosis (61); however, those subjects are outside the scope of this review.

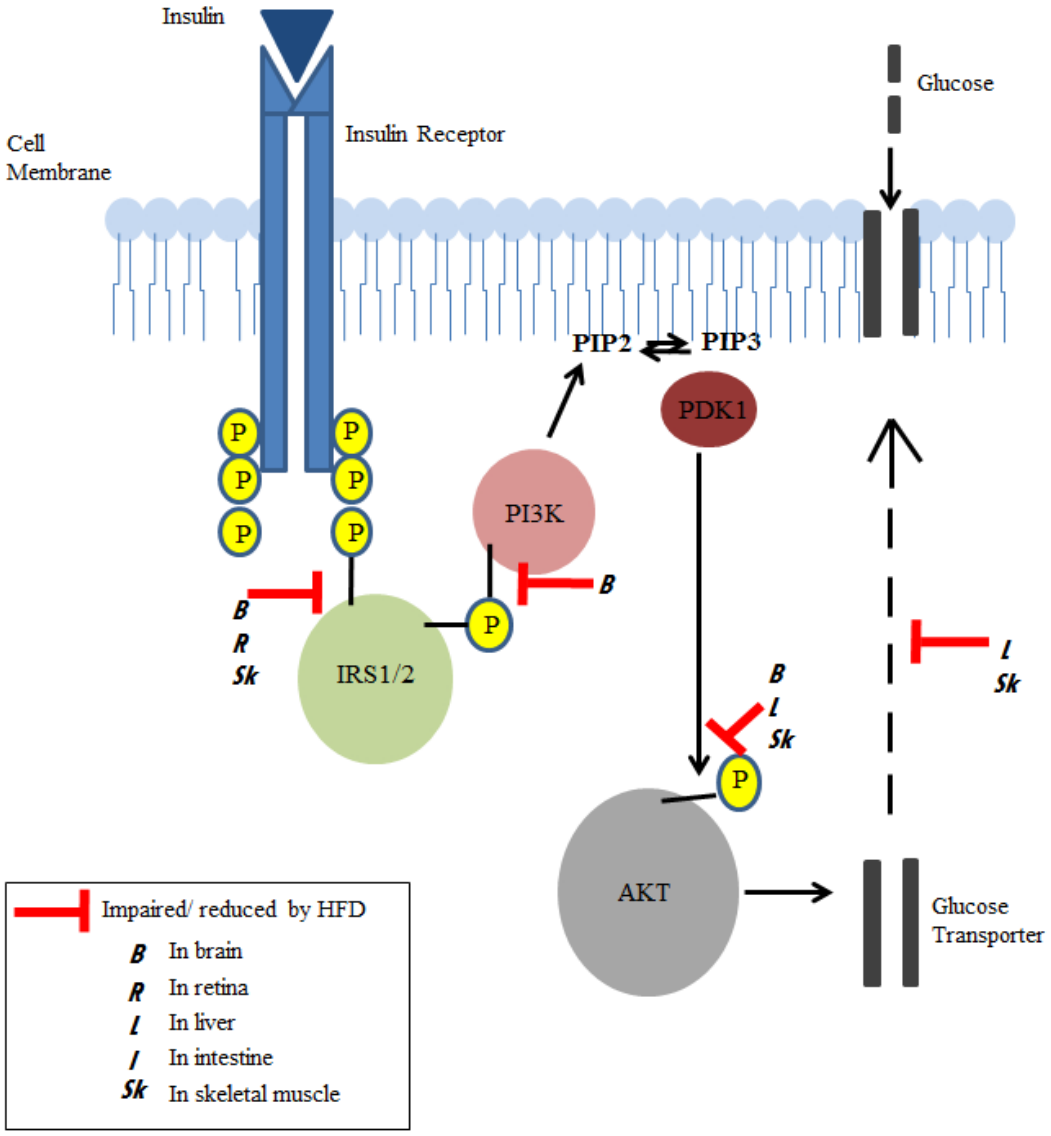

Figure 3. HFD-induced inhibition of insulin signaling.

Inflammation and insulin resistance/T2DM

Several pro-inflammatory mechanisms have been implicated in the development of obesity-related insulin resistance including macrophage infiltration and stimulation of multiple cytokine signaling pathways. Increased tissue macrophages not only contribute

to the production of inflammatory cytokines but have also been shown to exacerbate insulin resistance, as evidenced by improved insulin sensitivity with inhibition of macrophage infiltration (17,62). c-Jun-N-terminal kinase 1 (JNK1), a serine/threonine protein kinase, is a major cell signaling molecule also involved in obesity-induced insulin resistance. Studies have shown increased JNK1 expression in liver, muscle, intestine and adipose tissue during diet-induced obesity (63,64), as well as illustrated its impairment of the insulin signal via direct inhibition of IRS (65). JNK1 can be stimulated by cytokines such as TNFα (63) and IL-6 (66), as well as regulate the onset of obesity itself (67). In this way it seems JNK may serve as a central player in a vicious cycle of metabolic dysfunction that culminates in insulin resistance in many tissues. In fact, inhibition of JNK1 has been shown to prevent insulin resistance (63).

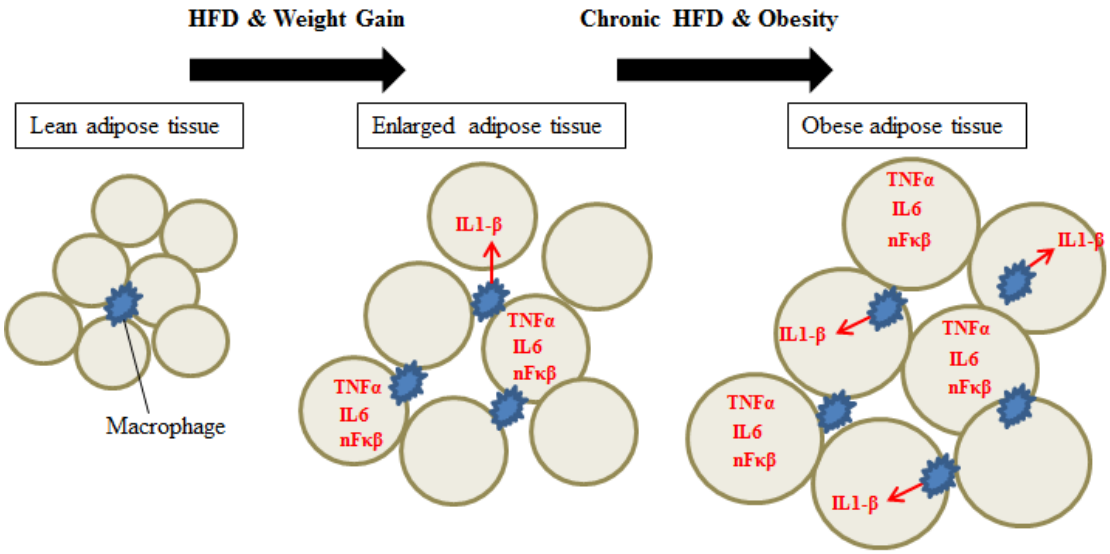

Figure 4. Obesity-induced inflammation in adipose tissue [modified from 56 and 108].

The generation of I kappa beta kinase beta (Ikkβ) is also known to contribute to insulin resistance (24). Similar to JNK1, Ikkβ also leads to serine phosphorylation (i.e. inhibition) of IRS1 and directly impairs the insulin signal cascade (68). Further, chronic production of Ikkβ, often seen in adipose tissue and liver of obese animals (69), activates NFκB both by direct stimulation of transcription factors and inhibition of NFκB inhibitors. Increased NFκB contributes to moderate insulin resistance (70) as well as the production of other inflammatory cytokines including TNFα and IL-6. Increased production of TNFα is especially damaging as it is shown to inhibit IRS-1 and decrease the tyrosine kinase activity of the IR (71). Therefore, the Ikkβ cascade seems to both directly impair insulin signaling and indirectly contribute to inflammation-induced insulin resistance. Another inflammatory signaling pathway associated with insulin resistance is the toll-like receptor (TLR) cascade. TLRs are transmembrane proteins that belong to the interleukin-1 family of receptors. They are found in intestinal epithelial cells, dendritic cells and in macrophages within almost all tissues and serve to initiate an immune response to pathogens, other stress stimuli such as lipopolysaccharides (LPS), and/or NFκB. TLR4, a widely expressed TLR in mammals, stimulates the MyD88 signaling pathway which results in the activation of mitogen-activated protein kinase (MAPK), NFκB, and the inflammatory cytokine transforming growth factor beta (TGFβ; 72,73). TLR4 plays a role in the development of obesity-related insulin resistance via stimulating the pro-inflammatory kinases JNK and Ikkβ after its activation by dietary fatty acids, which are elevated during overnutrition- and/or HFD-induced obesity. These cytokines and the inflammatory signaling cascades they are associated with play a particularly important

role in intestine-derived inflammation. In fact, recent research has focused on how these pathways converge in intestine and their direct role in driving intestinal insulin resistance.

INTESTINE INFLAMMATION AND INSULIN RESISTANCE

Intestine structure and physiological role

The intestine is an organ within the digestive tract that includes the small intestine, cecum, colon and rectum. Normal functioning within the small intestine especially, where nutrients are absorbed and first assimilated into the body, is vital to health as malabsorption and nutrient deficiencies can lead to severe metabolic complications. The small intestine is also responsible for recycling and producing some cholesterol (74) but more importantly chylomicrons and lipoprotein particles (75,76) which are essential for transporting fats throughout the body. Therefore, impairment to normal small intestine metabolism could be detrimental to fat distribution and/or lipid metabolism in other organs. The colon, as the last segment of the large intestine, is primarily responsible for reabsorbing fluids (e.g. water) and eliminating waste products from the body. However, the colon is the site where the majority of commensal bacteria are found and also serves as an important organ for flora-aided fermentation. Many nutrients necessary for metabolism such as short chain fatty acids are generated through such processes, and thus, normal colon function is required for the maintenance of overall health. Much research has investigated the underlying causes of abnormal intestine function and now implicates inflammation as a primary casual factor. Increased inflammation is associated with multiple disorders of the gastrointestinal tract (GI) including inflammatory bowel diseases

(IBD) such as ulcerative colitis (77), and colon cancer (78). Chronic local inflammation in the intestine is known to contribute to systemic inflammation and the generation of metabolic diseases (79).

Generation of intestine inflammation

Several disease states can lead to the development of inflammation in the small intestine. Bacterial, viral or parasitic infections (80-82), as well as physical damage to the small intestine can contribute to increased inflammation as the responding immune system involves macrophage recruitment and T-cell activation, both of which stimulate inflammatory cytokine pathways. Severe disorders such as Crohn's disease can also contribute to significant inflammation within the gut (83). Crohn's disease is thought to be an auto-immune type of disorder in which the body's immune system attacks the dietary components or normally occurring bacteria within the small intestine. Although the body's own cells and tissues are not targeted, this response leads to increased inflammation nonetheless which can be localized in both the lining and deeper tissue layers of the intestinal wall. Much research also implicates obesity in inflammatory cytokine production in the small intestine. Diet-induced models of obesity, particularly overnutrition/HFD feeding studies using saturated fats, are especially useful to induce an obese phenotype relatively quickly because saturated fats are stored very compactly within adipose tissue and thus, are harder to oxidize later, and are more pro-inflammatory compared to unsaturated fats (84-86). Results from such overnutrition studies show that chronic HFD feeding directly contributes to the generation of inflammation in the small

intestine through increased TLR4 expression (87), phosphorylation of JNK1 (64) and Iκκβ (24), and mRNA levels of IL-6 and TNFα (64). Interestingly, TLR4 and NFκB expressions are also increased after only short-term HFD (88). In addition, HFD-induced obesity also seems to indirectly contribute to small intestinal inflammation via changes in the gut microbial composition. Specifically, several studies have demonstrated a shift in the ratio of *Firmicutes* to *Bacteriodetes* populations (89,90). This shift leads to increased LPS production, which generates inflammation locally through stimulation of the TLR4 signaling pathway, and can contribute to systemic inflammation and worsen the obese phenotype (91). HFD-induced dysbiosis is also associated with reduced levels of intestinal alkaline phosphatase (IAP; 87) which normally functions to detoxify LPS. Changes in microbiota could also alter short chain fatty acid (SCFA) concentrations which, when lacking, may promote small intestinal inflammation. For example, butyrate treatment promotes IAP expression in intestinal cell lines (92) which is associated with protective effects on intestinal epithelial cells (IECs), and can prevent HFD-induced obesity (93). However, alterations of the gut microbiota could lead to reductions in bacterial metabolic ability and possibly, reduced butyrate production. Collectively, these findings indicate that diet-induced obesity contributes to small intestinal inflammation via both direct and indirect mechanisms.

Inflammation-induced intestinal damage

Intestinal inflammation can be especially detrimental to health as it interferes with normal intestinal metabolism and nutrient absorption. In fact, inflammation in the small

intestine can impair both micro- and macronutrient assimilation. For example, increased IL-6 expression, as seen in patients with Crohn's disease, is associated with reduced iron absorption (94). Further, long-term HFD feeding is associated with down regulation of numerous absorptive mechanisms in the small intestine. Most notably, membrane transporters including the Na,K-ATPase and GLUT2, both located on the basolateral membrane of IECs, are significantly reduced with HFD (13). Transporters for copper and amino acids, and cotransporters for SCFA are down regulated as well (13). Therefore, mineral, SCFA and amino acid absorption, as well as glucose assimilation into the body, can be significantly impaired in the small intestine after HFD.

Inflammation-induced intestinal insulin resistance

It is now well established that inflammation generated within the DIO phenotype can significantly contribute to insulin resistance in the small intestine. Overnutrition with saturated fat can be particularly causative (95) as saturated fat can stimulate multiple pro-inflammatory mechanisms compared to unsaturates. For instance, TLR4 expression, which is shown to be a key factor in the development of insulin resistance in many insulin-sensitive tissues including WAT and liver, is increased in response to high saturated fat intake in small intestine as well. In fact, TLR4 knockdown in many cell types and tissues can prevent the onset of insulin resistance induced by HFD/ increased FFAs (96-98). Although these studies have thus far been limited to muscle, adipose tissue, and macrophage, it seems plausible that TLR4 knockdown in intestine/ IECs would result in the same outcome. The interactions between HFD and bacterial populations with the

intestinal tract are also associated with insulin resistance. For example, mice with conventional bacteria fed a HFD express significantly higher levels of TNFα compared to germ-free mice fed the same diet (99). Further, it has been shown that short term HFD feeding leads to the translocation of commensal bacteria from the intestine to adipose tissue, which contributes to the development of inflammation and HFD-induced systemic hyperglycemia (100). Thus, the intestine both directly and indirectly contributes to the development of systemic insulin resistance within the overnutrition/DIO model, as it not only exhibits impaired insulin signaling but also serves as a mediator for the onset of insulin resistance in distal tissues. TNFα is also implicated in the onset of intestinal insulin resistance. Specifically, increased TNFα locally impairs the phosphorylation of both insulin receptor beta and IRS1 (101). Increased TNFα in the intestinal environment also results in increased phosphorylation of p38 MAPK, extracellular signal-related kinase1/2, and JNK, all of which have been implicated in the induction of insulin resistance (102). In conclusion, it appears that the development of insulin resistance within the intestine is caused by multiple mechanisms. Although some diseases states can culminate in the same outcome, the DIO phenotype appears to be especially causative as more than one mechanism is commonly stimulated.

PFKFB3/iPFK2

Physiological role

6-phosphofructo-2-kinase/ fructose-2,6-bisphophatase (PFKFB) is a glycolytic gene located on human chromosome 10 that codes for the enzyme inducible 6-

phosphofructo-2-kinase (iPFK2). iPFK2 serves to regulate glycolysis by driving the conversion of fructose-6-phosphate to fructose-2,6-bisphosphate (Figure 5) which is a powerful activator of phosphofructokinase 1 (PFK1), the enzyme driving the rate limiting step of glycolysis (103). Several isoforms of the PFKFB gene exist and are tissue specific (104,105). PFKFB1 is primarily expressed in the liver and skeletal muscle; PFKFB2 is fairly abundant throughout the body but is expressed highest in the thyroid gland; PFKFB4 expression is relatively low compared to other isoforms but seems to be expressed most in the testis. PFKFB3 is exceedingly abundant in tissues such as bone marrow, small intestine, and WAT, making it a useful tool for widespread research on impaired glucose metabolism. Further, the action of PFKFB3/iPFK2 is involved in lipogenesis and triglyceride synthesis in adipocytes (106). Thus, much research has focused on how altered expression of PFKFB3/ iPFK2 impairs lipid metabolism and contributes to the onset of obesity.

PFKFB3/iPFK2 and overnutrition

Interestingly, HFD-induced obese mice with an adipocyte-specific heterozygous knockdown of PFKFB3 ($PFKFB3^{+/-}$) show less weight gain and significantly increased rates of lipolysis as compared to wild-type (WT) controls (107). Reduced weight gain in $PFKFB3^{+/-}$ mice was attributed to less adipose fat mass and indicates that PFKFB3/iPFK2 plays a vital role in the onset of adiposity. However, loss of PFKFB3 exacerbates the generation of inflammation in adipose tissue, as evidenced by increased TLR4, TNFα, and IL-6 mRNA levels, as well as insulin resistance (107). Therefore, while PFKFB3 may

seem to be a potential therapeutic target for the prevention of obesity, the increased inflammation and insulin resistance caused by its reduction may actually be more harmful to health than increased adiposity. Researchers are now referring to this idea as 'healthy obesity', in which animal models or human patients are classified as obese but do not display the severity of the usually associated systemic inflammation or development of metabolic disorders. In fact, numerous studies have now provided empirical evidence to support this concept (108-110). Indeed, PFKFB3 overexpression has been shown to induce this phenotype. Specifically, while HFD-fed mice with targeted overexpression of PFKFB3/iPFK2 in adipose tissue did exhibit increased adipose tissue mass and hepatic steatosis, they had reduced adipose and liver inflammatory responses as well as improved insulin sensitivity as compared to WT controls (111). Therefore, it seems PFKFB3 plays an important role in regulating systemic insulin resistance in DIO animal models.

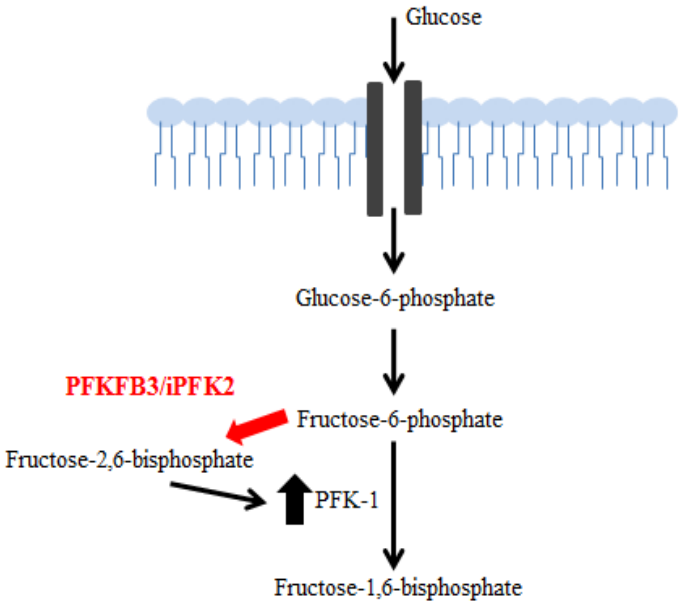

Figure 5. Role of PFKFB3/iPFK2 in stimulating glycolysis.

Intestinal PFKFB3/iPFK2

It is known that PFKFB3 expression is increased in small intestine in response to chronic HFD feeding (64); however, the extent to which PFKFB3 regulates intestine inflammation and insulin resistance is unclear. Further, it is unknown how PFKFB3 is regulated in IECs, the primary absorptive cell within the small intestine. The studies presented within this dissertation provide the first insight into the specific macronutrient regulation of PFKFB3, as well as its role as a potential link between metabolic and inflammatory signaling in small intestine.

SUMMARY

Obesity is now a health epidemic given that it affects a growing percentage of the population worldwide. It is considered as such and is especially dangerous because it is highly associated with the development of many metabolic disorders including some forms of cancer, cardiovascular disease, and T2DM. Therefore, continued research is necessary to further explore and understand the complex mechanisms underlying both its onset and contribution to other metabolic diseases. T2DM, which is characterized by insulin resistance and hyperglycemia, can be caused by prolonged malnutrition, i.e. overnutrition or excess fat intake, as increased FFAs damage pancreatic beta cells and impair normal lipolysis. T2DM is also very dangerous as it can impair normal metabolic pathways in the liver, skeletal muscle, and adipose tissue, as well as contribute to reduced nutrient absorption within the small intestine. It commonly occurs with obesity primarily because of the increased production of inflammatory cytokines induced by the obese phenotype.

The generation of inflammatory cytokines is normally a protective mechanism that is part of the body's innate immune system. However, with prolonged stress stimuli, such as long-term overnutrition with a HFD, the persistent production of cytokines can lead to an inflamed phenotype. Indeed, this is why obesity is characterized by chronic, low-grade systemic inflammation. Inflammation is known to contribute to insulin resistance through several mechanisms including increased macrophage infiltration and increased stimulation of multiple cytokine signaling pathways such as JNK, Iκκβ, and TLR cascades. These pathways not only directly interfere with normal insulin signaling but also

stimulate the production of inflammatory cytokines that can then interrupt the insulin signal. These underlying mechanisms of obesity-induced insulin resistance are confirmed in multiple tissues, including the small intestine. Normal functioning of the small intestine, and in particular the IECs, is essential for health as it is the primary site of nutrient absorption in the body. Damage to or diseases of the small intestine are therefore dangerous as normal nutrient assimilation can be interrupted. Overnutrition/DIO can also interfere with nutrient absorption is it leads to alterations in the microbiota, and increased production of proinflammatory cytokines. The DIO-associated inflammation is especially detrimental to health because it can significantly interfere with normal insulin signaling within the intestine and contribute to local and systemic insulin resistance/T2DM. Multiple mechanisms can underlie the development of insulin resistance, but inflammation generated from the DIO phenotype has been primarily implicated in many cell types and tissues, including intestine.

The gene PFKFB3 is a key regulator of glycolysis and is also shown to play a role in the onset of adiposity. However, it seems its most significant contribution to health may be in regulating the generation of inflammation. In fact, overexpression in adipocytes results in reduced local and systemic inflammation and sustained insulin sensitivity. Thus, PFKFB3 is also an important gene for preventing insulin resistance. While this result is confirmed in multiple cell types, to date its role in IECs has not been investigated. Further, how PFKFB3 expression is influenced by macronutrients within this cell type is unknown. Findings from such research would not only add to the current understanding of this gene

and its role in the onset of obesity-induced inflammation and insulin resistance, but could also lead to the development of targeted interventions for such metabolic complications.

CHAPTER III

GLUCOSE AND PALMITATE DIFFERENTIALLY REGULATE PFKFB3/iPFK2 AND INFLAMMATORY RESPONSES IN MOUSE INTESTINAL EPITHELIAL CELLS

INTRODUCTION

It is well established that inactivity and overnutrition are major determinants in the development of obesity and contribute to obesity-related metabolic diseases such as type 2 diabetes, fatty liver disease, and atherosclerosis (112-116). High saturated fat intake is an especially causative factor as it is known to directly contribute to the growth of individual adipocytes (117,118) which results in impaired lipid storage abilities and the generation of inflammation locally (119) and systemically in chronic conditions (120). It is now accepted that the obesity-associated chronic, low-grade systemic inflammation is a major underlying factor for the development of many metabolic diseases (121-124). As such, much research has investigated the mechanisms of diet-induced inflammation, particularly in adipose tissue.

The intestine has recently been implicated as another key organ that critically contributes to the development of obesity-associated chronic inflammation and systemic insulin resistance and metabolic dysregulation (125-127). While investigating how nutrient overload interacts with the intestine to cause systemic inflammation, a number of studies have shown that feeding a high-fat diet (HFD) to mice alters the composition of

the gut microbiota and leads to increased intestinal permeability (111,128). This in turn increases the levels of endotoxin in the intestinal lumen and circulation, thereby accelerating obesity and its related metabolic dysregulation. A recent study even indicated a role for HFD-induced intestinal eosinophil depletion, not inflammation, in contributing to defective barrier integrity and the onset of metabolic disease (128). Considering that the intestine is responsible for digestion, absorption, and assimilation of nutrients and that the nutrients absorbed by intestine have also undergone metabolism whose dysregulation accounts for increased proinflammatory responses, intestinal cells, in particular intestinal epithelial cells (IEC), may respond to nutrient overload to regulate its own inflammatory status prior to regulating inflammatory responses in distal organs. Indeed, in a mouse model of diet-induced obesity (DIO), feeding a HFD was shown to activate nuclear factor kappa B (NFκB) activity in intestine cells of the small intestine (64). While showing a similar finding in whole small intestine of HFD-fed mice (111), the study by Guo et al. further indicated that the anti-inflammatory responses in the intestine accounted for, at least in part, the insulin-sensitizing effect of peroxisome proliferator-activated receptor gamma (PPARγ) activation (123). Given this, there is a need to address the responses of IECs to nutrient overload in order to better understand the mechanisms of obesity-associated inflammation.

In the intestine, the gene 6-phosphofructo-2-kinase/fructose-2,6-bisphophatase 3 (PFKFB3) is abundantly expressed (64,111). As the product of PFKFB3, inducible 6-phosphofructo-2-kinase (iPFK2) generates fructose-2,6-bisphophate. The latter, as the most powerful activator of 6-phosphofructokinase-1, stimulates glycolysis. Recently

studies by Huo and Guo et al. have further demonstrated that PFKFB3/iPFK2 critically determines the balance of metabolic fluxes through glycolysis and fatty acid oxidation, thereby the generation of reactive oxygen species and proinflammatory responses in adipocytes (122,123). Unlike its role in adipose tissue/adipocytes, the role for PFKFB3/iPFK2 in the small intestine is less known. A previous study by Guo et al. showed increased gene and protein expressions in the small intestine in response to HFD feeding, as well as increased inflammation (64). However, the regulation of PFKFB3 specifically within IECs in relation to the inflammatory responses in IECs has not been investigated. Therefore, this study sought to first determine how diet/macronutrients influence PFKFB3/iPFK2 expression in IECs and secondly, how this relates to the IEC inflammatory status.

METHODS

Animal experiments

C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed under a 12 hr light/dark cycle with free access to water and fed *ad libitum*. At 5 – 6 weeks of age, male mice were fed either a low-fat diet (LFD) or HFD for 12 weeks. LFD and HFD consisted of 10% and 60% calories from fat, respectively. Both diets are products of Research Diets, Inc (New Brunswick, NJ). The complete macro- and micronutrient composition of the diets is shown in Table 1. During the feeding regimen, body weight and food intake were monitored weekly. After the feeding regimen, the mice were fasted for 4 hr before sacrifice for collection of blood and tissue samples (131). Some

mice were fasted similarly and used for insulin and glucose tolerance tests and/or IEC isolation as described below. All study protocols were approved by the Institutional Animal Care and Use Committees of Texas A&M University.

Table 1. Macro- and micronutrient composition of low-fat and high-fat diets

	LFD		HFD	
Macronutrients	g%	kCal%	g%	kCal%
Protein	19.2	20	26	20
Carbohydrates	67.3	70	26	20
Fat	4.3	10	35	60
TOTAL		100		100
kCal/ g	**3.85**		**5.24**	
Ingredients				
Casein	200	800	200	800
Corn Starch	315	1260	0	0
Sucrose	350	1400	68.8	275
Cellulose	50	0	50	0
Soybean Oil	25	225	25	225
Lard	20	180	245	2205
Mineral Mix S10026	10	0	10	0
Vitamin Mix V10001	10	40	10	40

Insulin and glucose tolerance tests

Insulin and glucose tolerance tests were performed as previously described (130,131). Briefly, the mice were fasted 4 hr and received an intraperitoneal injection of insulin (0.5 U/kg body weight for LFD-fed mice and 1 U/kg body weight for HFD-fed mice) or D-glucose (2 g/kg body weight). For insulin tolerance tests, blood samples (5 μl)

were collected from the tail vein before and at 15, 30, 45, and 60 min after the bolus insulin injection. Similarly, for glucose tolerance tests, blood samples were collected from the tail vein before and at 30, 60, 90 and 120 min after the glucose bolus injection.

Isolation of primary IEC

After the feeding regimen, the mice were sacrificed and the small intestine removed and cleaned of fecal debris. A small portion of the ileum was prepared for mRNA and protein analyses. The remaining intestine was first flushed with warm DMEM cell culture medium (Sigma-D5523; containing 4 mM L-glutamine, 4.5 g/l glucose, 1.5 g/l sodium bicarbonate, 10% FBS and 1% penicillin/streptomycin) before being placed in warm medium and transferred to a biosafety cabinet. The intestine was cut into 3 – 4, ~ 1.5-inch sections and inverted over bamboo splints. The splints were incubated at 37 ºC in DMEM + 2 mM EDTA for 10 min, with gentle shaking every 3 minutes. After incubation the splints were discarded, the medium filtered through a 70-µm filter, and centrifuged at 2,000 rpm for 5 min at 4 ºC. The medium was removed and the IEC pellets re-suspended in 3 ml warm DMEM medium and divided into 2, 1.5 ml tubes. The small tubes were then centrifuged at 3,000 rpm for 5 min at 4ºC. The medium was removed and the cell pellets re-suspended in 300 µl lysis buffer or 500 µl STAT-60 for protein and mRNA analyses, respectively. All samples were stored at -80 ºC until analysis.

Cell culture and treatment

The mouse-derived IEC line, CMT-93 (passage 10 – 30), was purchased from the American Type Culture Collection (ATCC, Catalog # CRL 223) and grown to confluence in DMEM (Sigma-D5523; containing 4 mM L-glutamine, 4.5 g/l glucose, 1.5 g/l sodium bicarbonate, 10% FBS and 1% penicillin/streptomycin) in 100-mm cell culture dishes in a humidified 5% CO_2 atmosphere at 37 ºC, as suggested by the manufacturer. Confluent cells were digested and split to 60-mm cell culture dishes and conditioned in low glucose (LG; 5.5 mM) medium for 24 hr prior to treatment. Thereafter, cells were incubated in LG or high glucose (HG; 27.5 mM) DMEM and treated with or without palmitate (50 μM) for an additional 24 hr. Bovine serum album (BSA) treatment was used as a control. After the treatment regimen, the cells were harvested for protein and mRNA and stored at -80 ºC for further analyses.

RNA isolation, reverse transcription, and real-time PCR

Total RNA was isolated from IECs and cultured CMT93 cells. Reverse transcription was performed using the GoScript™ Reverse Transcription System (Promega) and real-time PCR analysis was performed using SYBR Green (LightCycler® 480 system; Roche Life Science, Indianapolis, IN). The mRNA levels were analyzed for PFKFB3, interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα), and toll-like receptor 4 (TLR4). All primers were purchased from Integrated DNA Technologies. A total of 0.1 μg RNA was used for the determination. Results were normalized to 18s ribosomal RNA

and plotted as relative expression to the average of expression in LG-treated cells or cells of LFD-fed mice, which was set as 1.

Western blot analysis

Lysates were prepared from frozen IEC samples and cultured cells. Western blot analyses were performed as previously described (107). Protein amount of iPFK2 (Proteintech Group, Catalog # 13763-1-ap), c-Jun N-terminal kinase (JNK, p46; Santa Cruz, Catalog # sc-571), and phosphorylated-JNK (pJNK, Pp46; Santa Cruz, Catalog # sc-6254) was examined. The maximum intensity of each band was quantified using ImageJ software. Ratios of Pp46/p46 were normalized to GAPDH (Santa Cruz, Catalog # sc-25778) and adjusted relative to the average of control LFD-fed IEC or LG-treated control cells, which was arbitrarily set as 1 (AU).

Gene transcription reporter assay

A luciferase reporter assay was performed following methods previously described (133). Briefly, a reporter construct in which the luciferase expression is driven by an empty promoter (pGL3) or PFKFB3 promoter (pGL3-PFKFB3) was transfected into CMT-93 cells. After transfection for 24 hr, the cells were incubated with LG or HG medium and treated with or without palmitate (50 μM) for an additional 24 hr. Cell lysates were prepared and used to measure luciferase activity using a kit from Promega (Madison, WI). The luciferase activity was normalized to protein concentrations and adjusted relative to the average of LG- and BSA-treated pGL3 control, which was arbitrarily set as 1 (AU).

Statistical analysis

Numerical data are presented as means ± SE (standard error). Two-tailed ANOVA or Student's *t* tests were used for statistical analyses. Differences were considered significant at the $P < 0.05$.

RESULTS

Induction of obesity-related insulin resistance and glucose intolerance

To investigate nutritional regulation of IEC PFKFB3/iPFK2 expression and inflammatory responses in the context of obesity and insulin resistance, the present study fed C57BL/6J mice a HFD for 12 weeks. Compared with LFD-fed control mice, HFD-fed mice gained much more body weight (Fig. 6A; $P < 0.01$) after only several weeks on the respective diet. During the feeding regimen, diet groups consumed comparable amounts of food, but HFD-fed animals displayed a significant increase in feeding efficiency, which was calculated as milligram BW gained/Kcal consumed (Fig. 6B). Along with obesity, HFD-fed mice displayed insulin resistance even though they received twice the amount of insulin compared with LFD-fed mice (Fig. 6C) as indicated by the results from insulin tolerance tests. HFD-fed mice also showed impairment of glucose tolerance (Fig. 6D). As such, obesity-related insulin resistance was successfully induced in these mice.

Reduction of PFKFB3/iPFK2 in primary IECs

HFD feeding induces intestinal inflammation, which likely contributes to the development of systemic insulin resistance (126,127). Although HFD feeding increases iPFK2 amount in the intestine, it remains unknown the extent to which the PFKFB3/iPFK2 expression in primary IECs is altered. The present study examined PFKFB3/iPFK2 expression in primary IECs isolated from DIO- and control mice. Compared with those in IECs of LFD-fed mice, the mRNA levels of PFKFB3 in IECs of HFD-fed mice were decreased significantly (Fig. 7A; $P < 0.05$). Consistently, the amount of iPFK2 was reduced in primary IECs isolated from HFD-fed mice compared with those from LFD-fed mice (Fig. 7, B and C). Thus, HFD feeding decreased PFKFB3/iPFK2 expression in IECs, which is opposite to the effect of HFD feeding on increasing PFKFB3/iPFK2 in intestine extracts (64). The latter includes various types of cells.

Stimulation of proinflammatory responses in primary IECs

PFKFB3/iPFK2 displays anti-inflammatory properties (107,111,123). The present study examined if decreased IEC PFKFB3/iPFK2 expression is associated with increased proinflammatory responses. Relative to that in primary IECs of control mice, the proinflammatory signaling through JNK1 was increased in primary IECs of DIO mice (Fig. 8, A and B). In addition, the mRNA levels of proinflammatory cytokines, e.g., IL-6 and TNFα, in IECs of DIO mice were significantly higher than in IECs of control mice (Fig. 8C). Similar changes were also observed in the mRNA levels of TLR4 (Fig. 3C), whose activation promotes proinflammatory responses.

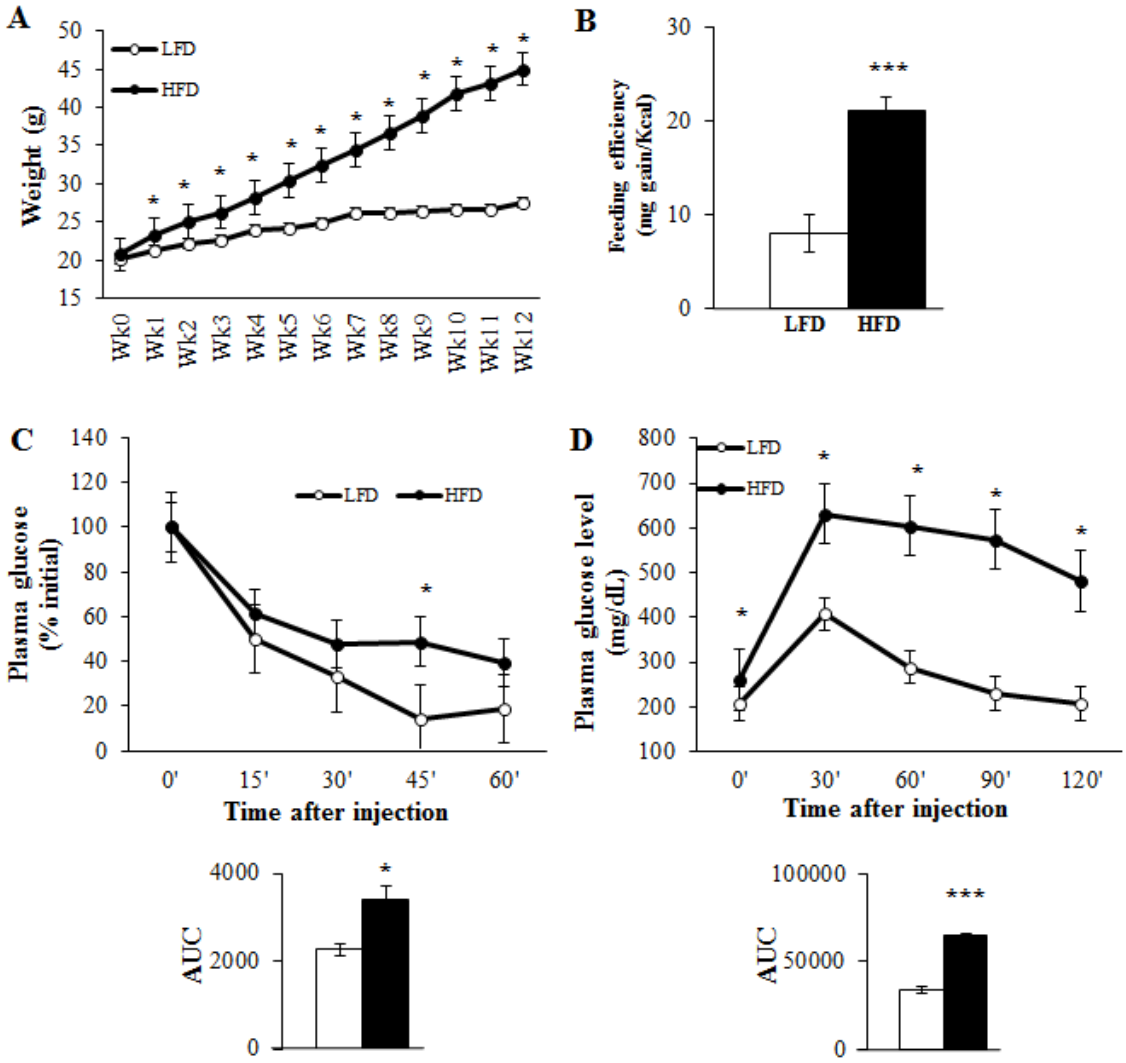

Figure 6 HFD induction of obesity-related insulin resistance and glucose intolerance. Male C57BL/6J mice, at 5 – 6 weeks of age, were fed a high-fat diet (HFD) or low-fat diet (LFD) for 12 weeks. n = 10. (**A**) Body weight. (**B**) Feeding efficiency was calculated by normalizing food intake to body weight. (**C**) Insulin tolerance tests. After the feeding regimen, the mice were given a bolus intraperitoneal injection of insulin (1 U/kg for HFD-fed mice and 0.5 U/kg for LFD-fed mice). (**D**) Glucose tolerance tests. For C and D, areas under curves (AUC) were calculated based on the corresponding tolerance tests. For A – D, data are means ± SE. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ HFD vs. LFD (AUC in C and D) for the same time point (A, C, and D).

Together, these results suggest that HFD feeding increased IEC proinflammatory responses, which were associated with a decrease in PFKFB3/iPFK2 expression in IECs.

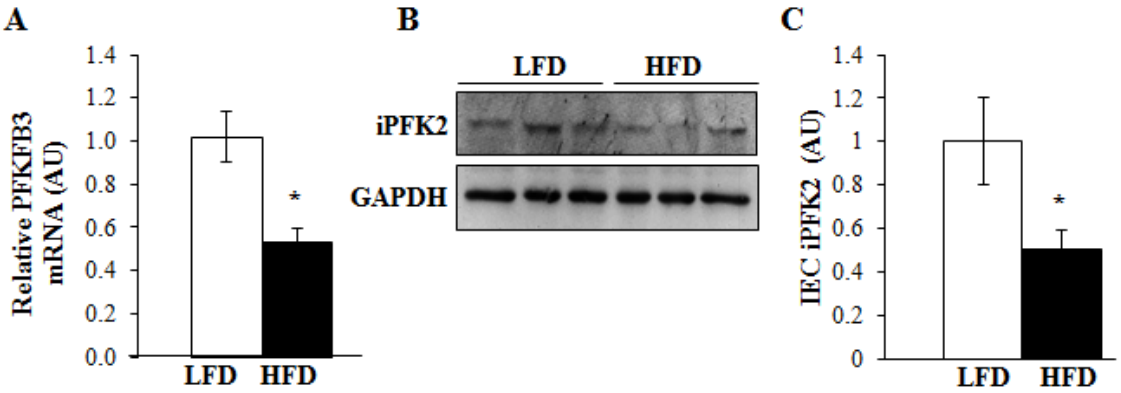

Figure 7 Dietary effects on IEC PFKFB3/iPFK2 expression. Male C57BL/6J mice, at 5 – 6 weeks of age, were fed a high-fat diet (HFD) or low-fat diet (LFD) for 12 weeks. After the feeding regimen, mice were anesthetized and subjected to isolation of primary IECs. (**A**) IEC expression of PFKFB3 mRNAs was determined using real-time PCR. (**B**) Western blot analysis of IEC iPFK2 amount. (**C**) Quantification of IEC iPFK2. For bar graphs, data are means ± SE, n = 4 – 6. *, $P < 0.05$ HFD vs. LFD.

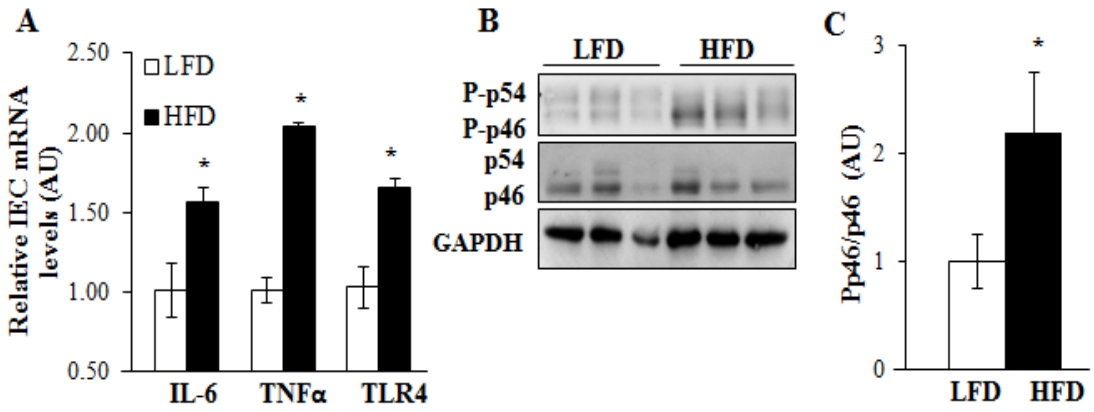

Figure 8 Dietary effects on IEC proinflammatory responses. Male C57BL/6J mice, at 5 – 6 weeks of age, were fed a high-fat diet (HFD) or low-fat diet (LFD) for 12 weeks. After the feeding regimen, mice were anesthetized and subjected to isolation of primary IECs. (**A**) Western blot analysis of IEC JNK signaling. (**B**) Quantification of IEC Pp46/p46. (**C**) IEC expression of IL-6, TNFα, and TLR4 mRNAs was determined using real-time PCR. IL-6, interleukin-6; TNFα, tumor necrosis factor alpha; and TLR4, toll-like receptor 4. For B, data are means ± SE, n = 4 – 6. *, $P < 0.05$ HFD vs. LFD for the same gene.

Direct effects of glucose and palmitate on IEC PFKFB3/iPFK2 expression and proinflammatory responses

As indicated by the above results, overnutrition decreased PFKFB3/iPFK2 expression and increased proinflammatory responses in primary IECs. To gain the nutritional insight, the present study examined the direct effects of glucose and palmitate, two major macronutrients associated with overnutrition, on IEC responses. When the effects of glucose were examined, treatment of CMT-93 cells with 27.5 mM glucose increased iPFK2 amount relative to treatment of CMT-93 cells with 5.5 mM glucose, indicating a stimulatory effect of glucose on PFKFB3 expression (Fig. 9, A and B). In contrast, palmitate appeared to mainly account for increased proinflammatory responses

in cultured CMT-93 cells. In the presence of low glucose, palmitate did not alter JNK1 signaling. However, in the presence of high levels of glucose, palmitate treatment caused a significant increase in JNK1 signaling (Fig. 9, A and B). In addition, palmitate treatment caused significant increases in the mRNA levels of IL-6 and TNFα, as well as TLR4 in the presence of either low or high levels of glucose (Fig. 9C).

Stimulatory effect of glucose on PFKFB3 transcription

The present study further explored the effect of glucose on stimulating IEC PFKFB3 expression. In a time course study, treatment of CMT-93 cells with low levels of glucose for 24 hr did not alter the mRNA levels of PFKFB3 compared with treatment of CMT-93 cells with low levels of glucose for 4 hr (Fig. 10A). However, in the presence of high levels of glucose, treatment of CMT-93 cells for 24 hr significantly increased the mRNA levels of PFKFB3 relative to treatment of CMT-93 cells for 4 hr (Fig. 10A). Furthermore, high levels of glucose remained a dominant effect in stimulating PFKFB3 expression even in the presence of palmitate, which appeared to decrease the mRNA levels of PFKFB3 (Fig. 10B). To gain the mechanistic insight of the stimulatory effect of glucose, a reporter assay was performed and showed that high levels of glucose increased the transcription activity of the PFKFB3 promoter (Fig. 10C). Unlike glucose, palmitate treatment did not alter the transcription activity of the PFKFB3 promoter.

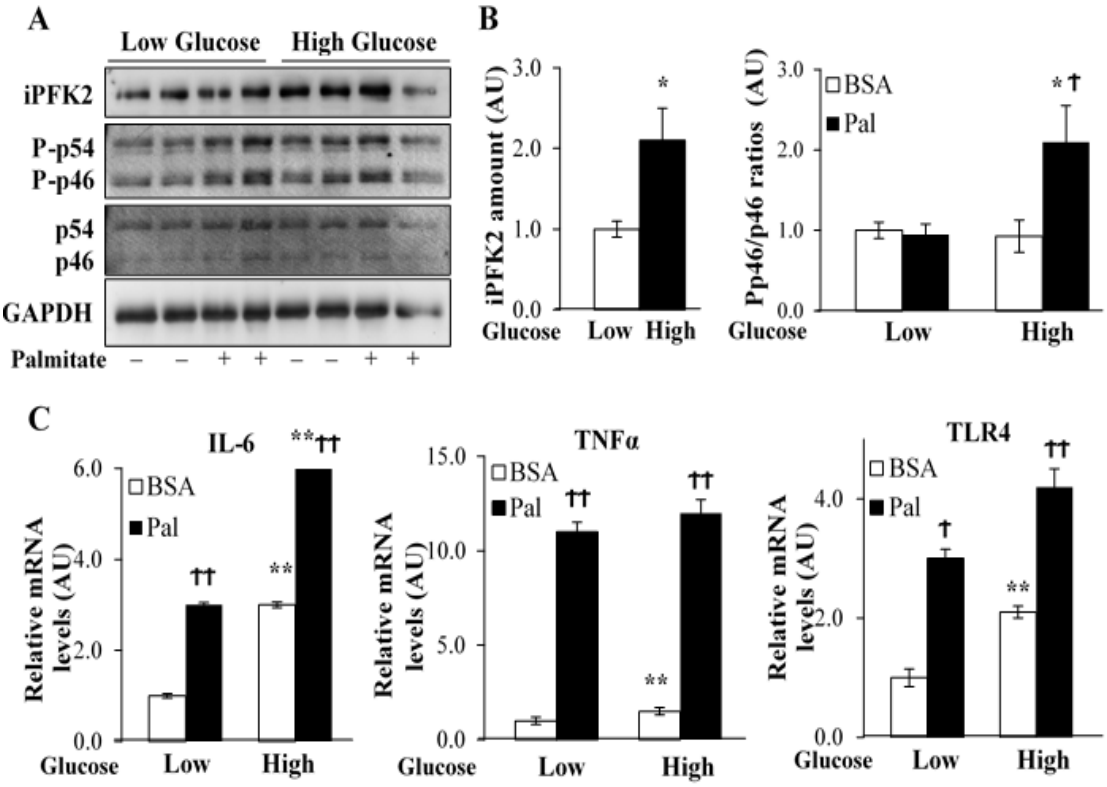

Figure 9 Effects of glucose and palmitate on IEC iPFK2 and proinflammatory responses. CMT-93 cells were treated as described in methods. (**A**) Western blot analyses of IEC iPFK2 amount and JNK signaling. (**B**) Quantification of IEC iPFK2 and Pp46/p46. (**C**) IEC expression of IL-6, TNFα, and TLR4 mRNAs was determined using real-time PCR. iPFK2, inducible 6-phosphfructo-2-kinase; IL-6, interleukin-6; TNFα, tumor necrosis factor alpha; and TLR4, toll-like receptor 4. For A and B, CMT-93 cells were treated with low glucose (5.5 mM) or high glucose (27.5 mM) in the presence of palmitate (Pal, 50 μM) or bovine serum albumin (BSA) for 24 hr. For B and C, data are means ± S.E. n = 4. *, $P < 0.05$ and **, $P < 0.01$ High glucose vs. Low glucose (B) for the same gene (C); †, $P < 0.05$ and ††, $P < 0.01$ Pal vs. BSA for the same condition (low or high in B and C).

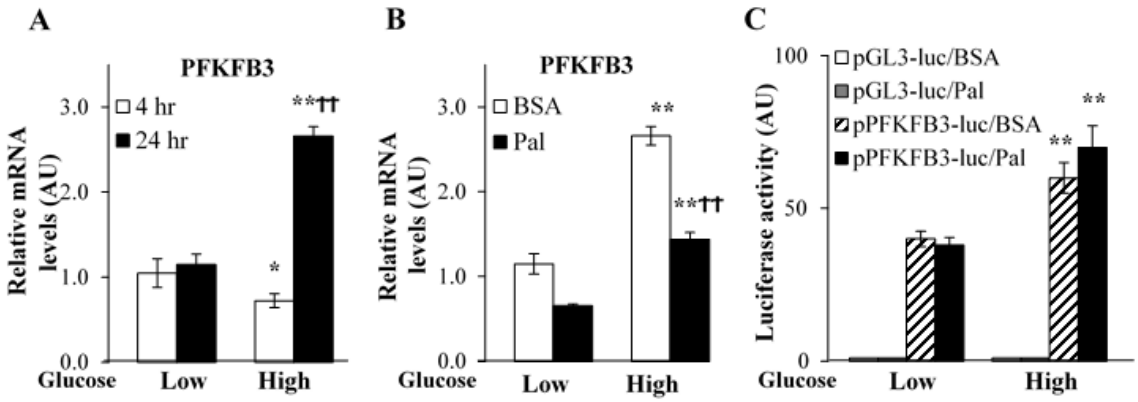

Figure 10 Effects of glucose and palmitate on PFKFB3 gene transcription. CMT-93 cells were treated as described in methods. Data are means ± S.E. n = 4 – 6. (**A**) Time course and dose responses of glucose regulation of PFKFB3 expression. (**B**) Palmitate regulation of PFKFB3 expression. (**C**) Regulation of PFKFB3 transcription. For A and B, CMT-93 cells were treated with low glucose (5.5 mM) or high glucose (27.5 mM) for 4 hr or 24 hr (A) in the presence of palmitate (Pal, 50 μM) or BSA for 24 hr (B). *, $P < 0.05$ and **, $P < 0.01$ vs. High glucose vs. Low glucose for the same time (A) or same treatment (B); ††, $P < 0.01$ 24 hr vs. 4 hr (A) or Pal vs. BSA (B) within the same treatment. For C, CMT-93 cells were transfected with pGL3-PFKFB-luc or a control reporter construct (pGL3-luc) and treated with LG or HG in the presence or absence of palmitate (50 mM) for an additional 24 hr. **, $P < 0.01$ High glucose vs. Low glucose for the same treatment (pGL3-PFKFB-luc/BSA or pGL3-PFKFB-luc/Pal).

DISCUSSION

Recent studies have established that PFKFB3/iPFK2 links nutrient metabolism and inflammatory responses in several tissues and cell types, e.g., adipocytes and endothelial cells (107,111,123,132). In the intestine, PFKFB3/iPFK2 has also been implicated as a regulator that critically controls the development of intestinal inflammation during obesity (64,107). Significantly, PFKFB3/iPFK2 is involved in the effect of rosiglitazone, one of the only two currently prescribed medicines as insulin-sensitizers for the treatment of type 2 diabetes, on suppressing intestinal inflammation (123). The current study presented here

builds upon this finding by investigating PFKFB3/iPFK2 specifically within IECs, a topic which has not been previously studied.

In DIO mice, the mRNA levels of PFKFB3 and the amount of iPFK2 were significantly decreased in IECs compared with their respective levels in IECs from LFD-fed mice. Surprisingly, these changes were opposite to the previous finding that the iPFK2 amount was increased in intestine extracts of DIO mice (64). Considering that the intestine includes various types of cells, it is possible that HFD increased PFKFB3/iPFK2 in cells other than IECs, and those cells had higher abundance of PFKFB3/iPFK2 than IECs. Additionally, it is possible that during and after the digestion, absorption, and metabolism of nutrients, the composition of nutrients and the metabolites of nutrients were different across IECs and cells other than IECs. As a result, IECs and cells other than IECs likely interacted, respectively, with different nutrients and/or metabolites, thereby displaying differential consequences on PFKFB3/iPFK2. While these possibilities need to be further examined, it is important to consider that LFD provides a significantly high amount of carbohydrates (i.e. corn starch). Subsequently, IECs from this diet group would display increased expressions of PFKFB3/iPFK2 and thus, exhibit reduced levels in diets with less carbohydrate stimuli (i.e. HFD). This outcome is evident in both PFKFB3 mRNA and iPFK2 amount in primary IECs. As additional evidence, glucose and palmitate showed differential effects on PFKFB3/iPFK2 expression in cultured cells (see below). To be noted, the markers of proinflammatory responses however were significantly higher with HFD. This effect appears to be due to the high amount of saturated fat, e.g., palmitate, in the diet, which is known to induce proinflammatory responses in many tissues/cells

(133,134). In fact, the findings from cultured IEC cell line studies further confirm this postulation as evidenced by increased JNK1 signaling and the mRNA levels of several pro-inflammatory markers in response to palmitate treatment. Therefore, palmitate appears to serve as the primary underlying factor as to why IECs display increased inflammatory responses with HFD feeding. Of importance, the status of proinflammatory responses in IECs was reversely correlated with PFKFB3/iPFK2 expression, suggesting that PFKFB3/iPFK2 also has an anti-inflammatory role in IECs.

To better explain the effect of diet *in vivo*, the present study investigated the effects of individual major macronutrients on PFKFB3/iPFK2 expression. The results indicated that dietary components exert differential effects on PFKFB3/iPFK2. Notably, glucose, at high levels, significantly increased the mRNA levels of PFKFB3 and the amount of iPFK2. This stimulatory effect of glucose was expected as PFKFB3 is highly involved in the generation of fructose-2,6-bisphophate when carbohydrates, in particular glucose, are in excess. In fact, the role of PFKFB3/iPFK2 in this manner has been demonstrated in numerous cell types (106,135). Mechanistically, glucose stimulation of PFKFB3/iPFK2 expression was attributable to the effect of glucose on increasing the transcription activity of the PFKFB3 promoter. In support of this, high levels of glucose significantly increased the activity of luciferase whose expression was driven by a 6.1 kb fragment of the PFKFB3 promoter. A previous study by Sans et al. had shown that the PFKFB3 promoter region contains the CACGTG-containing regulatory elements that interact with MondoA:Mlx complex (136). The latter has been shown to mediate the effect of glucose on stimulating the expression of a number of genes that participate in glycolysis and lipogenesis when

nutrients are in excess. Therefore, within IECs glucose acts to directly stimulate PFKFB3/iPFK2 expression. This data further validates the high PFKFB3/iPFK2 levels seen in LFD-fed mice.

As a critical component of HFD, palmitate has been previously shown to serve as an endogenous ligand to the TLR4 in IECs and therefore directly stimulated the expression of proinflammatory cytokines via the TGF-β and NF-κB pathways (133). Consistently, the present study showed that palmitate treatment significantly increased TLR4 and proinflammatory cytokine expression in cultured IECs. Of interest, palmitate treatment also decreased the mRNA levels of PFKFB3 and the amount of iPFK2. However, this effect of palmitate was not sufficient to counter against the effect of glucose on increasing PFKFB3/iPFK2. Nonetheless, this finding further confirmed that the HFD-induced decrease in PFKFB3/iPFK2 expression in primary IECs was due to low levels of carbohydrates (glucose) relative to LFD. In addition, consistent with the results from primary IECs of HFD-fed mice, the decrease in PFKFB3/iPFK2 also correlated well with increased proinflammatory responses in cultured IECs. Based on these findings, it is likely that one mechanism for palmitate to increase IEC proinflammatory responses was to decrease PFKFB3/iPFK2 expression, thereby leading to a decrease in the anti-inflammatory effect.

It should be pointed out that the HFD-induced decrease in PFKFB3/iPFK2 expression in the primary IECs also was reversely correlated with systemic insulin resistance and dysregulation of glucose homeostasis. Based on this relationship, it is likely that PFKFB3/iPFK2 in IECs participates in the regulation of obesity-associated insulin

resistance and dysregulation of glucose homeostasis. Furthermore, the role played by PFKFB3/iPFK2 appears to be attributable to the anti-inflammatory properties of PFKFB3/iPFK2 as this is indicated by the results of the present study in both primary IECs and cultured CMT-93 cells and by the findings of previous studies in adipocytes (111,123). Although future study is needed to validate a specific role for the PFKFB3/iPFK2 in IECs in the control of obesity-associated insulin resistance and metabolic dysregulation, targeting PFKFB3/iPFK2 in IECs through nutritional intervention could offer novel approaches for prevention and/or treatment of obesity-associated metabolic diseases.

In summary, this study provides evidence for the first time that major macronutrients, e.g., glucose and palmitate, differentially influence the expression of PFKFB3/iPFK2 within IECs. As outlined in the proposed scheme (Figure 6), glucose directly stimulates PFKFB3/iPFK2 expression whereas palmitate more so contributes to the generation of inflammation. The present study also provides the insight into the potential role for PFKFB3/iPFK2 in protecting against the proinflammatory responses within IECs. Overall, diet has a significant impact on PFKFB3/iPFK2 expression within IECs in the context of obesity-associated inflammation. Because of this, activating PFKFB3/iPFK2 in IECs through nutritional approaches could be beneficial for obesity-associated metabolic diseases.

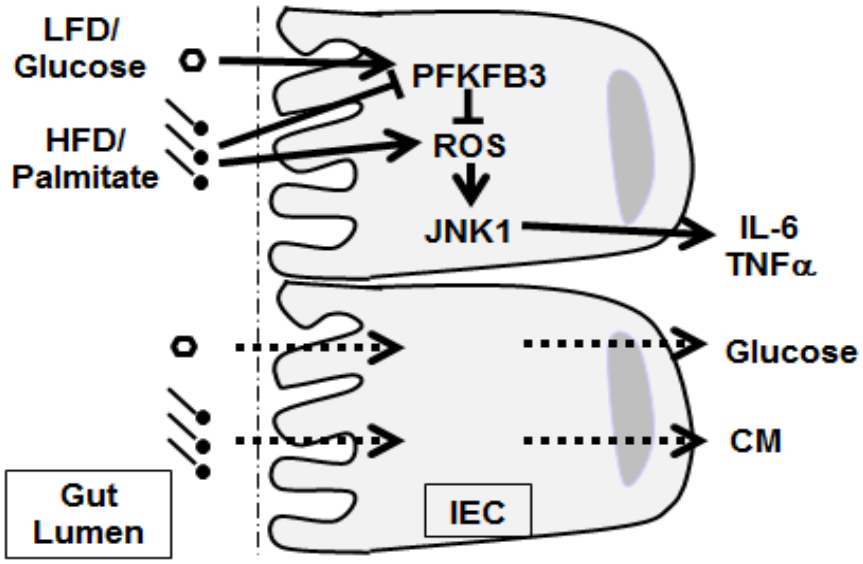

Figure 11 PFKFB3/iPFK2 mediates nutritional control of IEC inflammatory responses. The proposed schematic diagram summarizes the differential effects of major macronutrients, e.g., glucose and palmitate on PFKFB3/iPFK2 expression and the inflammatory responses within IECs. Upon LFD feeding, glucose, as a major macronutrient at high concentration, stimulates PFKFB3/iPFK2. This in turn inhibits the proinflammatory responses in IECs, likely through the established effect of PFKFB3/iPFK2 on suppressing the generation of ROS. Upon HFD feeding, the stimulatory effect on PFKFB3/iPFK2 is not present due to low concentrations of glucose. This in turn de-inhibits the proinflammatory responses in IECs. In addition, palmitate, as a major macronutrient of HFD, has a direct proinflammatory effect on IECs. The combined effects exacerbate IEC proinflammatory responses, which may contribute to HFD-induced systemic inflammation. IEC, intestinal epithelia cells; LFD, low-fat diet; HFD, high-fat diet; ROS, reactive oxygen species; JNK1, c-Jun N-terminal kinase 1; IL-6, interleukin-6; TNFα, tumor necrosis factor alpha; TLR4, toll-like receptor 4; and CM, chylomicrons.

CHAPTER IV

PFKFB3/iPFK2 EXPRESSION REGULATES PROINFLAMMATORY RESPONSES

INTRODUCTION

6-phosphofructo-2-kinase/fructose-2,6-bisphophatase (PFKFB) is a glycolytic gene located on human chromosome 10 that codes for the enzyme inducible 6-phosphofructo-2-kinase (iPFK2). iPFK2 serves to regulate glycolysis by driving the conversion of fructose-6-phosphate to fructose-2,6-bisphosphate, which is a powerful activator of phosphofructokinase 1 (PFK1), the enzyme responsible for the rate limiting step of the pathway (103). Several isoforms of the PFKFB gene exist (PFKFB1-4) but PFKFB3 is exceedingly abundant in tissues such as bone marrow, small intestine, and WAT (64), and is therefore a useful tool for widespread research on glucose metabolism in metabolic diseases such as obesity. Multiple factors are shown to influence PFKFB3/iPFK2 expression and/or activity including stress stimuli (137,138) and hormones (139,140), and data presented in the preceding chapter provides the first evidence for macronutrient regulation of PFKFB3/iPFK2 expression within intestinal cells (see chapter III).

Multiple studies have shown an inverse association between PFKFB3/iPFK2 amount and inflammatory responses in multiple cell types and tissues. For example, disruption of PFKFB3 in adipocytes is linked to increased expressions of proinflammatory

cytokines (122). Previous findings presented within this dissertation suggest the same relationship within intestinal epithelial cells (IECs). Specifically, an overnutrition-induced reduction of PFKFB3/iPFK2 is associated with increased mRNA and protein levels of the proinflammatory markers IL-6, TNFα, TLR4 and pNFκB (see chapter III). Other studies have further demonstrated that PFKFB3/iPFK2 critically determines the balance of metabolic fluxes through glycolysis and fatty acid oxidation, and thereby the generation of reactive oxygen species (ROS) and proinflammatory responses (111,122,123). In other words, increased PFKFB3/iPFK2 expression and/or activity accounts for increased rates of glycolysis and less beta oxidation and therefore, a reduced overall proinflammatory response.

This role of PFKFB3/iPFK2 has been extensively studied in adipose tissue; however, whether a direct cause and effect relationship between PFKFB3/iPFK2 and inflammatory responses exists in IECs is unknown. Previously, only associations have been suggested. Therefore, the present study investigated how overexpression of PFKFB3/iPFK2 relates to proinflammatory responses within IECs.

METHODS

Cell line culture and treatment

The mouse-derived IEC line, CMT-93 (passage 10–30), was purchased from the American Type Culture Collection (ATCC CRL 223) and grown to confluence in IMDM culture medium (Sigma-56479C; containing 4 mM L-glutamine, 25mM HEPES, 4.5 g/L glucose, 3.024 g/L sodium bicarbonate, 10% FBS and 1% penicillin/streptomycin) in

100mm cell culture dishes in a humidified 5% CO_2 atmosphere at 37ºC, as suggested by the manufacturer. To first determine inflammatory cytokine production in the presence of overexpression of PFKFB3/iPFK2, confluent cells were transfected with plasmids containing the cDNA of iPFK2 (Acg-PFKFB3) with Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Cells were also transfected with an empty vector as a control (GFP). Cells were then treated with LPS (100ng/mL) or PBS (control) and harvested and saved in -80ºC for protein and mRNA analyses. Additionally, the present study also investigated inflammatory marker expression with inhibition of PFKFB3/iPFK2. Cells were treated with the PFKFB3 inhibitor (3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one (3PO, 2µg/mL, Calbiochem, 525330) or DMSO (control) for 24 h. Cells were additionally treated with LPS (100ng/mL) or PBS (control) and harvested and saved as described above.

Nitroblue tetrazolium assay

A nitroblue tetrazolium (NBT) assay was conducted to investigate the role of PFKFB3/iPFK2 in regulating superoxide generation. Briefly, both PFKFB3/iPFK2 overexpression and inhibition *in vitro* experiments, described above, were repeated but in place of harvest, all cells were treated with 0.2% NBT (in PBS) and incubated for 90 min. Afterward all NBT liquid was discarded, and cells were washed with PBS and treated with 50% glacial acetic acid. Experimental dishes were gently rocked for 3 min, scraped with cell scrapers, and rocked an additional 3 min. All liquid was collected and subjected to sonification (3 pulses of 6 s) to completely dissolve the precipitate.

Following sonification all tubes were centrifuged at 2600 x g for 30 s and OD of the supernatant was determined at 560 nm.

Animals and experimental treatments

To investigate the extent to which PFKFB3/iPFK2 can regulate overnutrition-induced inflammation in IECs, the present study conducted a HFD feeding experiment using 5-6 week old male wild-type (WT) mice and mice with a heterozygous disruption of PFKFB3 ($PFKFB3^{+/-}$). Homozygous disruption of PFKFB3 results in embryonic lethality, so $PFKFB3^{+/-}$ mice were generated as previously described (141). All animals were housed at the Kleberg Animal and Food Science Center on the Texas A&M University campus and kept under a 12 hour light/dark cycle. Animals were given free access to water and fed a HFD *ad libitum* for 12 weeks. HFD consisted of 60%, 20% and 20% calories from fat, protein and carbohydrates, respectively. The full composition of the diet is shown above (Table 1, see chapter III). Body weight and food intake were monitored weekly. This study was approved and conducted in accordance with the Institutional Animal Care and Use Committee of Texas A&M University.

Primary IEC harvest

To determine the role of PFKFB3/iPFK2 in regulating the generation of inflammation *in vivo*, primary IECs were isolated and preserved from HFD-fed WT and $PFKFB3^{+/-}$ mice as described previously (see chapter III).

Glucose and insulin tolerance tests

Glucose and insulin tolerance tests were administered as previously described (123). Briefly, following a 4 hr fast, blood samples were collected from the tail vein before and 30, 60, 90 and 120 min after the bolus D-glucose (10ug/g) injection. For insulin tolerance mice received an intraperitoneal injection of insulin (1 U/kg) and blood was collected before and 15, 30, 45, and 60 min following. Plasma glucose concentration for both tolerance tests was assessed using a glucose assay kit (Sigma- GAHK20).

RNA isolation and real-time PCR

Total RNA was isolated from cultured CMT93 cells and primary IECs. Reverse transcription and real-time PCR were performed in the same manner as described in the above chapter. The mRNA levels were analyzed for PFKFB3, IL-6, TNFα, and TLR4. A total of .1 μg RNA was used for determination and results were normalized to 18s ribosomal RNA and plotted as relative expression to the average of expression in GFP-transfected or DMSO-treated cells, or HFD-fed WT mice, which were arbitrarily set as 1.

Western blot analysis

Lysates were prepared from cultured cells and frozen tissue samples. Western blot analyses were run as previously described (123). Protein expression for iPFK2 and JNK1 signaling was determined. Equal loading was confirmed using GAPDH.

Statistics

All western blot data was quantified using the NIH ImageJ software (version 1.48). Statistical significance was determined by unpaired, two-tailed ANOVA or student's t-test and set at the $P<0.05$ level. Numerical data are presented as the mean ± SE (standard error).

RESULTS

PFKFB3/iPFK2 expression modulates proinflammatory responses

The present study investigated the effect of PFKFB3/iPFK2 overexpression and inhibition on the generation of inflammation in IECs. Overexpression of PFKFB3/iPFK2 was associated with lower levels of JNK1 signaling (Fig. 12A). Further, rtPCR analyses showed similar results (Fig. 12B). Specifically, LPS-stimulated mRNA levels of all thee proinflammatory markers were significantly lower in cells overexpressing PFKFB3. PFKFB3 overexpression also resulted in increased superoxide production (Fig. 12C), indicated by increased NBT reduction. Therefore, *Pfkfb3* expression seems to modulate the severity of the inflammatory response induced by stress stimuli. In support of this finding, PFKFB3/iPFK2 inhibition was associated with higher levels of inflammation. Specifically, TNFa mRNA amount and NBT reduction were significantly greater when PFKFB3/iPFK2 was inhibited (Fig. 13A and B).

Overnutrition-induced obesity and insulin resistance

To determine the ability of PFKFB3/iPFK2 in modulating a diet-induced inflammatory response in IECs, the present study fed WT and PFKFB3$^{+/-}$ mice a HFD for 12 weeks. Interestingly, although PFKFB3$^{+/-}$ animals gained less body weight (Fig. 14A), they exhibited severe insulin resistance/glucose intolerance (Fig. 14C and D). In fact, although individual time points were similar within the glucose and insulin tolerance tests, both area under the curve analyses (AUC) indicated significantly higher plasma glucose levels in PFKFB3$^{+/-}$ animals. Thus, HFD feeding induces a more severe insulin resistant phenotype with PFKFB3 disruption. This phenotype was not induced by increased energy intake as food intake was similar between the two groups (Fig. 14B).

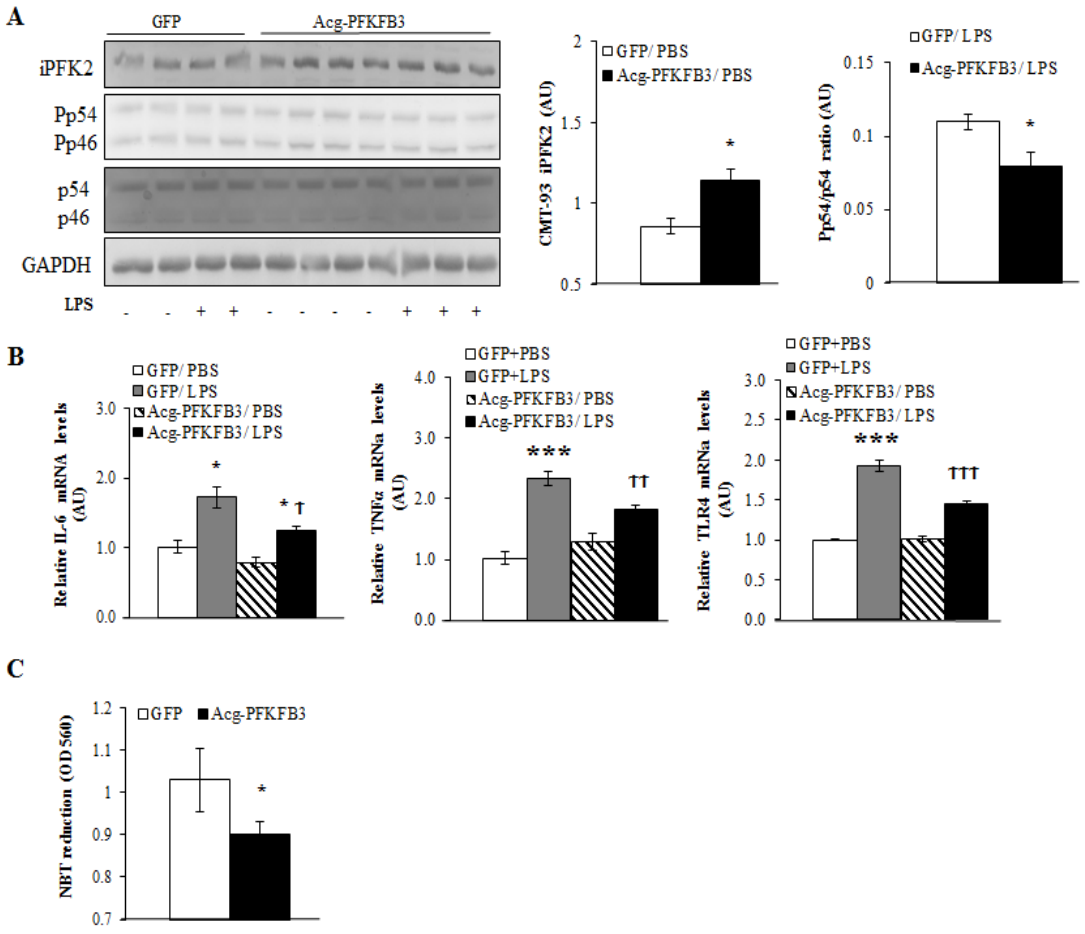

Figure 12 PFKFB3/iPFK2 overexpression results in reduced proinflammatory responses. CMT-93 cells were treated as described in methods. (**A**) Western blot analyses of PFKFB3/iPFK2 overexpression and quantification of IEC iPFK2 and Pp54/p54. (**B**) Effects of PFKFB3 overexpression on mRNA levels. (**C**) Effects of PFKFB3 overexpression on NBT reduction. For A – C, data are means ± SE, n = 4. *, $P < 0.05$ and ***, $P < 0.001$ Acg-PFKFB3 vs GFP (A,C) or LPS vs PBS for the same promoter (B). †, $P < 0.05$; ††, $P < 0.01$; †††, $P < 0.001$ Acg-PFKFB3/ LPS vs GFP/ LPS (B). iPFK2, inducible 6-phosphfructo-2-kinase; Pp54, phosphorylated JNK1; p54, total JNK1; Il-6, interleukin-6; TLR4, toll-like receptor 4; TNFα, tumor necrosis factor alpha.

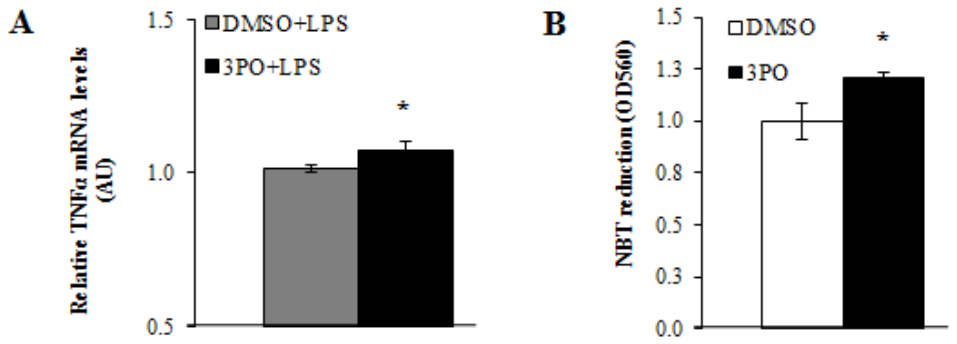

Figure 13 PFKFB3/iPFK2 inhibition is associated with increased proinflammatory responses. CMT-93 cells were treated as described in methods. (**A**) Effects of PFKFB3 inhibition on mRNA levels. (**B**) Effects of PFKFB3 inhibition NBT reduction. For A and B, data are means ± SE, n = 4. *, $P < 0.05$ and ***, $P < 0.001$ 3PO vs DMSO. TNFα, tumor necrosis factor alpha.

HFD-induced inflammatory response in PFKFB3$^{+/-}$ mice

Primary IECs from PFKFB3$^{+/-}$ mice displayed significantly higher inflammatory marker mRNA levels (Fig. 15). Specifically, both IL-6 and TLR4 mRNA amounts in heterozygous animals were much higher than levels found in HFD-fed WT animals. This finding not only supports *in vitro* results but provides evidence for the role of PFKFB3 in combating the generation of diet-induced inflammation specifically within IECs. Unfortunately, western blot protein analyses could not be conducted due to low primary IEC protein amount.

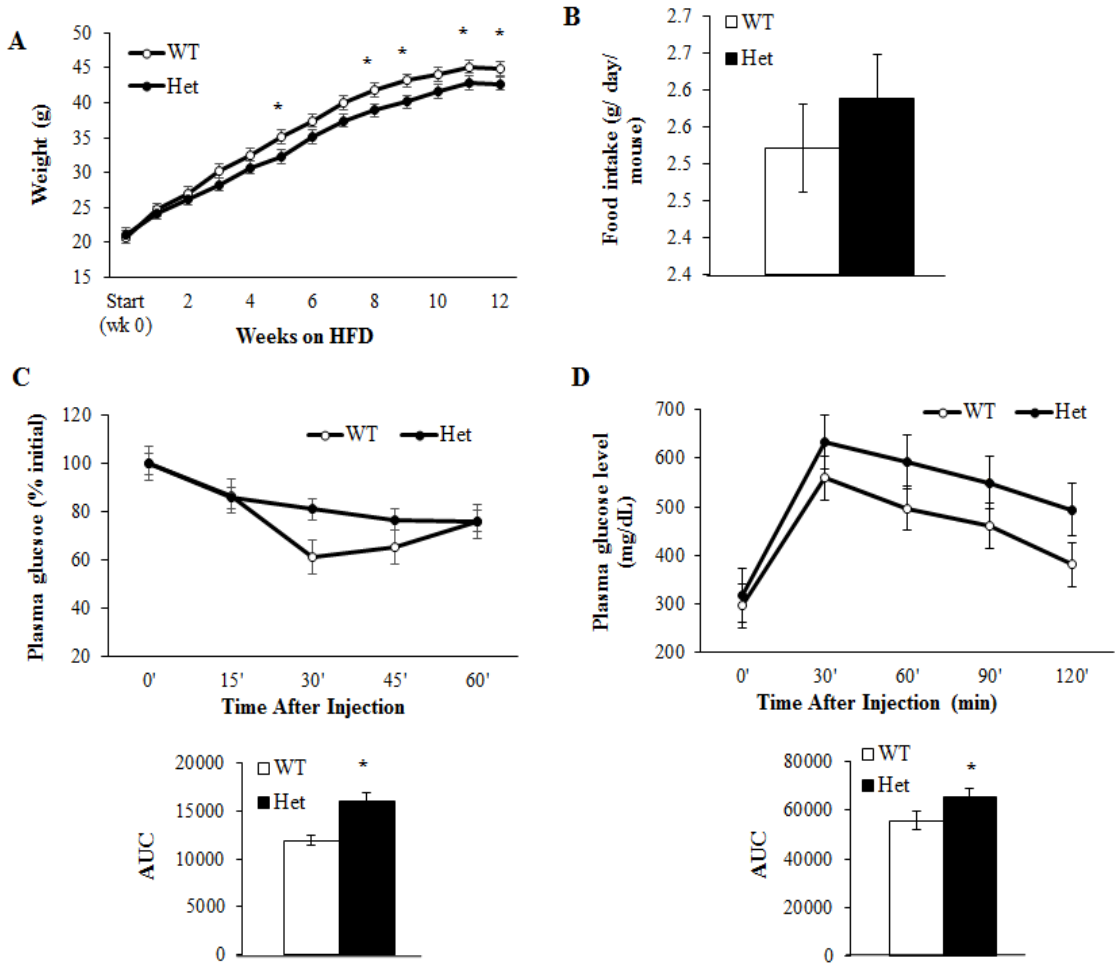

Figure 14 PFKFB3/iPFK2 disruption exacerbates HFD-induced insulin resistance. WT or PFKFB3$^{+/-}$ male C57BL/6J mice, at 5 – 6 weeks of age, were fed a HFD for 12 weeks, n = 10. (**A**) Body weight. (**B**) Food intake. (**C**) Insulin tolerance tests. (**D**) Glucose tolerance tests. For C and D, areas under curves (AUC) were calculated based on the corresponding tolerance tests. For A – D, data are means ± SE. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ HFD vs. LFD (AUC in C and D) for the same time (A, C, and D). HFD, high-fat diet.

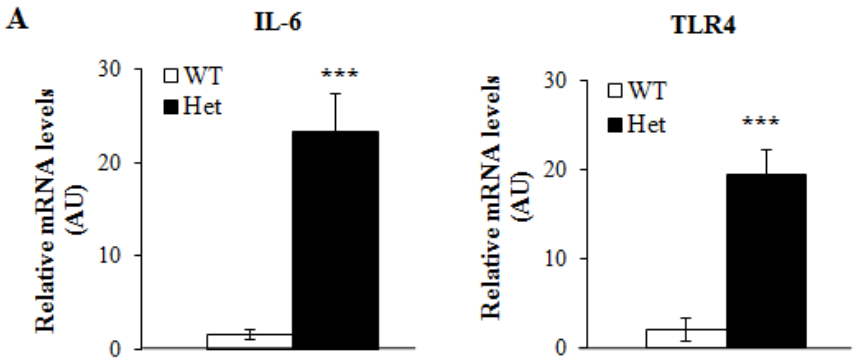

Figure 15 Increased inflammatory markers in primary IECs from HFD-fed PFKFB3$^{+/-}$ mice. Primary IECs were isolated from HFD-fed WT and PFKFB3$^{+/-}$ mice as described in methods. (**A**) Effects of PFKFB3 disruption on IEC mRNA inflammatory markers. ***, $P < 0.001$ HFD-fed PFKFB3$^{+/-}$ vs HFD-fed WT animals. Il-6, interleukin-6; TLR4, toll-like receptor 4.

DISCUSSION

The present study provides evidence for a direct, inverse relationship between PFKFB3 expression and proinflammatory responses within IECs. Additional data presented here further suggests that intestinal PFKFB3 expression may help control glucose dysregulation and proinflammatory mechanisms induced by HFD feeding. Together these findings support an anti-inflammatory action of PFKFB3/iPFK2 specifically within IECs, which has not been shown previously.

Overexpression of PFKFB3/iPFK2 within CMT-93 cells resulted in decreased levels of both protein and mRNA inflammatory markers, which supports an anti-inflammatory role of this gene within IECs. This finding is similar to that seen previously in studies investigating PFKFB3/iPFK2 within adipocytes. Specifically, adipocytes display decreased pNFκB protein amount as well as reduced mRNA levels of both IL-6

and TNFα when PFKFB3/iPFK2 is overexpressed (111). Further, the present study provides evidence that inhibition of PFKFB3/iPFK2 results in increased proinflammatory responses, indicated by increased mRNA amount of the inflammatory cytokine TNFα and increased production of superoxides. Therefore, PFKFB3/iPFK2 appears to play a similar role in regulating inflammation in multiple cell types throughout the body, including IECs.

Given the anti-inflammatory role of PFKFB3/iPFK2 suggested by *in vitro* data, the present study conducted a HFD-feeding experiment to determine the role of PFKFB3/iPFK2 in regulating diet-induced intestinal inflammation. As indicated by body weight and glucose tolerance data, all animals developed obesity and insulin resistance. However, AUC calculations indicate that HFD-fed PFKFB3-disrupted animals displayed significantly higher plasma glucose concentrations compared to HFD-WT mice. These data not only confirm the diet-induced phenotype, but suggest that PFKFB3/iPFK2 plays an important role in regulating systemic glucose levels. Indeed, previous studies have shown this same result (107). However, none have addressed the link between PFKFB3/iPFK2 expression and diet-induced inflammation within IECs. Interestingly, global disruption of PFKFB3/iPFK2 was associated with increased proinflammatory responses within IECs, as evidenced by increased mRNA amounts of IL-6 and TLR4. Unfortunately protein inflammatory markers in IECs could not be analyzed. These data are significant nonetheless as they are similar to previous reports. Specifically, Guo et al 2013 found increased mRNA expressions of TNFα and IL-6, and increased NFkB and JNK1 inflammatory signaling in the small intestine of HFD-fed PFKFB3$^{+/-}$ vs HFD-fed WT animals. It should be noted that the previous study analyzed the whole small intestine

and not individual cell types within. Therefore, results from the current study build upon the previous in that they shed light on the role of PFKFB3/iPFK2 specifically within the absorptive cells of the small intestine.

It has been shown that PFKFB3/iPFK2 regulates proinflammatory responses mainly through the balance of metabolic fluxes (111,122,123,142). Specifically, because of its powerful action on stimulating glycolysis, PFKFB3/iPFK2 expression drives substrates away from beta oxidation. In turn, less ROS are produced and less proinflammatory signaling pathways activated. Results presented in the current study support this mechanism. Overexpression of PFKFB3/iPFK2 would allow for increased rates of glycolysis, while disrupted PFKFB3/iPFK2 could greatly contribute to increased rates of beta oxidation. In summary, studies such as this are necessary to further understand how genes/proteins balance metabolic and inflammatory pathways in IECs, where nutrients and metabolites converge and first interact with host cells. Future experiments are needed to further validate this role of PFKFB3/iPFK2 and investigate if an intestine-specific deletion of PFKFB3/iPFK2 causes increased local inflammatory responses and possibly interferes with nutrient absorption and/or systemic metabolism.

CHAPTER V

INDOLE INFLUENCES PFKFB3/iPFK2 EXPRESSION AND ATTENUATES INFLAMMATION IN MURINE INTESTINAL EPITHIAL CELLS

INTRODUCTION

Within the intestine several factors are known to regulate PFKFB3/iPFK2, including insulin (139) and excessive stress stimuli such as reactive oxygen species (138). However, as a gene involved in regulating metabolism and more specifically glycolysis, nutrients have the most significant influence on its expression. In particular, glucose has the most profound effect on stimulating PFKFB3 (see chapter III), compared to saturated fats which seem to exert little to no direct effect (see chapter III). Indeed, previous studies have shown that the PFKFB3 promoter region contains the CACGTG-containing regulatory elements that interact with the MondoA:Mlx complex (136), which mediates the effect of glucose on stimulating the expression of a number of genes that participate in glycolysis. In addition to regulating glycolysis, PFKFB3/iPFK2 is also beneficial in the prevention of inflammation. In fact, PFKFB3/iPFK2 amount is inversely related to levels of inflammatory cytokines and proinflammatory transcription factors such as NF-κβ. This relationship is valid in multiple cell types (111,122), including IECs (see chapter III). Therefore, PFKFB3 is an important factor in the balance between normal metabolic function and stimulation of inflammatory pathways. This is particularly significant in intestine, where nutrients first interact with and are assimilated into the body. However, it

is unknown if other metabolic factors specific to the intestinal environment influence intestinal PFKFB3/iPFK2 expression.

Bacterial metabolites are especially interesting as alterations within the gut microbiome have been implicated in the onset of diet-induced metabolic disorders (91,143-145). In fact, microbial alterations are associated with reduced metabolite and/or peptide production (146,147) and impaired nutrient absorption (145). Therefore, the association between diet/overnutrition and bacterial metabolites and how this possibly translates to altered PFKFB3 expression is necessary to investigate as it could further explain the underlying mechanisms of inflammation following overnutrition.

Indole is a metabolite produced from the bacterial breakdown of tryptophan. All bacterium possessing a TnaA gene, and the subsequent ability to produce *tryptophanase*, are capable of producing indole (148). Thus far, it is known that over 80 species of bacteria within the intestinal environment produce indole (149), including both gram-positive and gram-negative types, as well as species from both dominant phyla, the *Bacteriododetes* and *Firmicutes*. Indole acts as a signaling molecule between bacterium and aids in biofilm and spore formation (150,151), plasmid stability and mobility (152), and virulence (153). Recently however indole has been shown to also play an important role in maintaining the integrity of the host gut epithelium. For example, Bansal et al. (154) demonstrated a strengthened mucosal barrier, as evidenced by increased expressions of genes involved in tight junction formation following exposure to indole. Further, IECs from indole-fed mice display significantly increased tight junction formation, expressions of molecules associated with adherens junctions, and a higher resistance to DSS-induced colitis (155).

Thus, indole appears to play a vital role in the overall function of the intestinal cell barrier. In addition, indole has been shown to exert anti-inflammatory properties on host cells, the mechanisms of which appear to be twofold. First, there is evidence supporting increased expression of genes involved in IL-10 production (154). At the same time, it seems indole inhibits TNF- mediated stimulation of NF-κB (154). Therefore, within the gut indole serves to simultaneously stimulate anti-inflammatory and inhibit proinflammatory pathways. However, research on the anti-inflammatory abilities of indole is in its infancy and other roles and mechanisms of action are likely. Therefore, a possible interaction between indole and PFKFB3 is worth investigating. Results from such studies could potentially help to further explain the mechanism by which indole exerts its anti-inflammatory ability, but also provide evidence for an interaction between PFKFB3 and metabolites, which until now has not been investigated. Thus, the purpose of this study was to determine if indole influences PFKFB3/ iPFK2 expression in IECs and investigate if such an interaction underlies another anti-inflammatory mechanism of indole.

METHODS

Cell line culture and treatment

To investigate interactions between indole and intestinal PFKFB3/iPFK2, the present study used the mouse-derived IEC line, CMT-93 (passage 10–30), purchased from the American Type Culture Collection (ATCC CRL 223) and grown to confluence in Iscove's Modified Dulbecco's medium (IMDM) culture medium (HyClone, cat no SH30005.02) in a humidified 5% CO_2 atmosphere at 37ºC, as suggested by the

manufacturer. Confluent cells were transferred to 6-well experimental plates and conditioned to medium containing 0.2 mM indole or dimethyl sulfoxide (DMSO, control) for 24 hours prior to treatment. Cells were then treated with LPS (100 ng/mL) or PBS (control) and harvested and saved in -80 ºC for protein and mRNA analyses. To determine any dose effect of indole confluent cells were treated with 0.1 mM, 0.2 mM or 0.4 mM indole or DMSO (control) for 24 hours. After the experimental period cells were harvested and saved for protein and mRNA analyses.

Gene transcription reporter assay

To determine if indole acts directly on PFKFB3, a luciferase reporter assay was performed following methods previously described (133). Briefly, a reporter construct in which the luciferase expression is driven by an empty promoter (pGL3-luc, control) or PFKFB3 promoter (pPFKFB3-luc) was transfected into CMT-93 cells. Following the 24 hr transfection period, cells were incubated with medium containing 0.1 mM, 0.2 mM indole, or DMSO for 6 and 24 hr. Cell lysates were prepared and used to measure luciferase activity using a kit from Promega (Madison, WI). The luciferase activity was normalized to protein concentrations and adjusted relative to the average of DMSO-treated pGL3 controls, which was arbitrarily set as 1 (AU).

Animals and experimental treatments

To further explore the interaction between indole and PFKFB3/ iPFK2 in the context of obesity, the present study conducted a HFD feeding experiment. 5 - 6 week old

male C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were housed at the Kleberg Animal and Food Science Center on the Texas A&M University campus and kept under a 12 hour light/dark cycle. Animals were given free access to water and fed a HFD ad libitum for a total of 16 weeks. HFD consisted of 60%, 20% and 20% calories from fat, protein and carbohydrates, respectively. The full composition of the diet is shown previously in Table 1. Animals were further divided into two treatment groups and dosed daily the last six experimental weeks via oral gavage with 50mg/kg indole dissolved in bovine serum albumin (BSA), or PBS in BSA (control). Body weight and food intake were monitored weekly. This study was approved and conducted in accordance with the Institutional Animal Care and Use Committee of Texas A&M University.

Glucose and insulin tolerance tests

Glucose and insulin tolerance tests were administered as previously described (129,130). Briefly, after fasting 4 hours mice received an intraperitoneal injection of D-glucose (10ug/g) or insulin (1 U/kg). Plasma glucose concentration for both tolerance tests was assessed using a glucose assay kit (Sigma- GAHK20).

Isolation of primary IECs

At the end of the experimental period all animals were sacrificed and the small intestine treated and primary IECs isolated as described above. Briefly, following

inversion of the small intestine and a short digestion period in EDTA, IEC cells were pelleted and saved at -80 ºC for both mRNA and protein analyses.

RNA isolation and real-time PCR

Total RNA was isolated from primary IECs and cultured CMT93 cells. Reverse transcription and real-time PCR were performed in the same manner as described above. The mRNA levels were analyzed for PFKFB3, IL1-β, and IL-6. A total of 0.1 μg RNA was used for determination and results were normalized to 18s ribosomal RNA and plotted as relative expression to the average of expression in DMSO-treated cells or PBS-dosed mice, which were arbitrarily set as 1 (AU).

Western blot analysis

Lysates were prepared from frozen cultured cells and IEC samples. Western blot analyses were run as previously described (130). Protein amount for iPFK2, NF-κB (p65, Cell Signaling, D14E12), phosphorylated-NF-κB (Pp65, Cell Signaling, S536), and TLR4 (Santa Cruz, sc-293072) was determined. The maximum intensity of each band was quantified using ImageJ software. Ratios of Pp65/ p65 were normalized to GAPDH and adjusted relative to the average control of DMSO-treated cells or PBS-dosed mice, which was arbitrarily set as 1 (AU).

Statistics

Numerical data are presented as the mean ± SE (standard error). Two-tailed ANOVA or student's t-test was used for statistical significance which was set at the $P<0.05$ level.

RESULTS

Stimulation of PFKFB3 and reduction of inflammatory cytokines in cultured CMT-93 cells

To explore any influence of indole on IEC PFKFB3/ iPFK2, the present study exposed CMT-93 cells to indole for 24 hours. Indeed, treatment of CMT-93 with 0.2 mM indole caused a significant increase in iPFK2 expression as compared to DMSO (Fig. 16, A and B). Real-time PCR analysis showing increased PFKFB3 mRNA amount supports this finding (Fig. 16C). Further, in LPS-stimulated conditions indole treatment significantly reduced levels of the inflammatory markers Pp65 and TLR4 (Fig. 16, A and B). Although previous studies have shown anti-inflammatory abilities of indole, to our knowledge, this is the first evidence showing this result specifically within IECs. Further, an association between indole and PFKFB3/iPFK2 expressions has not been shown previously.

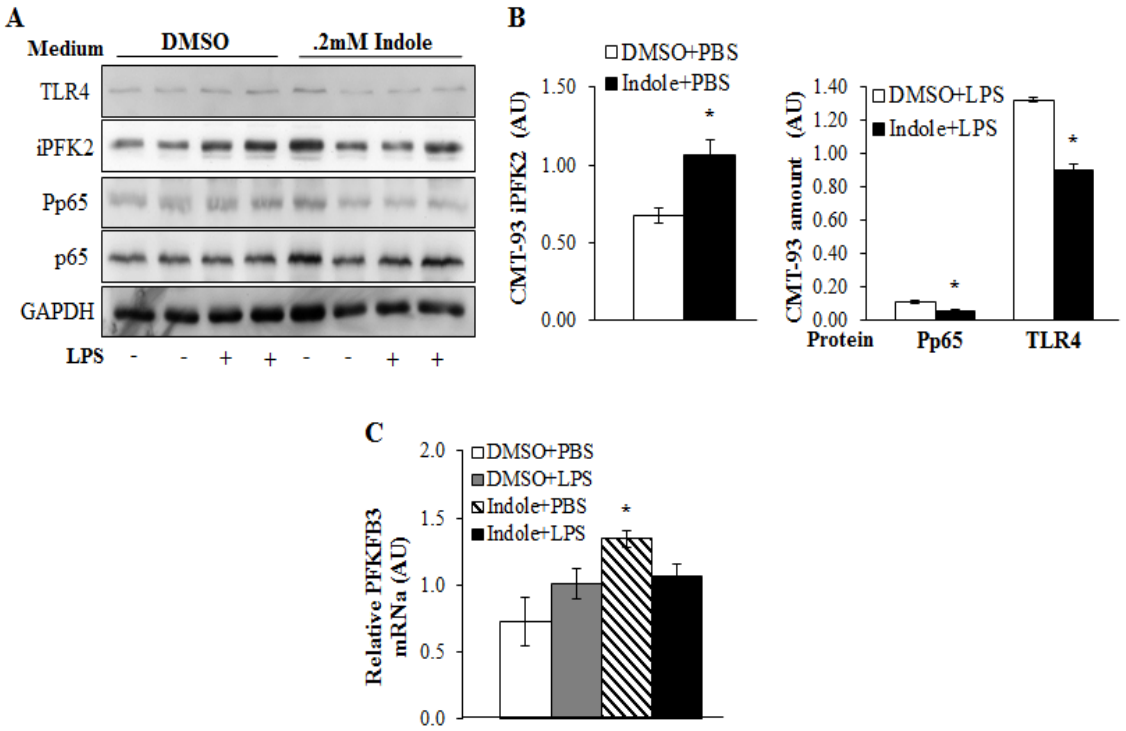

Figure 16 Indole stimulates IEC iPFK2 and reduces proinflammatory responses. CMT-93 cells were treated as described in methods. (**A**) Western blot analyses of IEC iPFK2 and TLR4 amounts, and NF-κβ signaling. (**B**) Quantification of IEC iPFK2, Pp65/p65, and TLR4. (**C**) IEC expression of PFKFB3 mRNAs was determined using real-time PCR. iPFK2, inducible 6-phosphfructo-2-kinase; and TLR4, toll-like receptor 4. For A and B, CMT-93 cells were treated with LPS (100mg/mL) or PBS (control) for 6 hr or 30mins for mRNA and protein analyses, respectively. For B and C, data are means ± S.E. n = 4. *, $P < 0.05$ and **, $P < 0.01$ .2mM indole vs DMSO for the same gene.

To investigate a dose-response effect of indole, CMT-93 cells were treated with DMSO or 0.1 mM, 0.2 mM, or 0.4 mM indole. Consistent with the previous *in vitro* results, 0.2 mM indole was associated with the greatest increase in iPFK2 amount (Fig. 17, A and B). In fact, indole appears to increase iPFK2 in a slight dose-dependent manner up to 0.2 mM, above which it becomes toxic to cells, evidenced by observed increases in

cell death. However, only the iPFK2 amount at the 0.2 mM concentration of indole was significantly different from that of the DMSO-treated cells. Also in line with previous findings, the inflammatory marker Pp65 was the lowest following treatment with 0.2 mM indole compared to other doses (Fig. 17, A and B). Pp65 amount at the 0.4 mM indole concentration was actually comparable to that of 0.2 mM; however, this is more than likely because less cells survive at such high concentrations of indole so any increased inflammatory response would not be seen. mRNA analyses indicate a strong dose effect of indole (Fig. 17C). Specifically, PFKFB3 levels increase in a dose-dependent manner and peak at a concentration of 0.2 mM. PFKFB3 levels at the 0.4 mM treatment, while still higher than that of control, are much less than those at the 0.2 mM concentration. The mRNA levels of the inflammatory markers IL1-β and IL-6 also demonstrate a slight dose-dependent response to indole treatment.

Increased PFKFB3 promoter activity in the presence of indole

Following *in vitro* experiments, the present study conducted a reporter assay to determine if indole has a direct effect on stimulating the promoter transcription activity of the PFKFB3 gene. PFKFB3 promoter activity in all 6 hr indole- or DMSO-treated pPFKFB3-luc cells was statistically similar. Excitingly however, results show significantly higher PFKFB3 promoter activity following 24 hr treatment of both 0.1 mM and 0.2 mM indole, as compared to pPFKFB3-luc cells treated for 24 hr with DMSO (Fig. 18). This result is especially noteworthy as it provides the first evidence supporting that

indole acts through increasing PFKFB3 promoter transcription activity to directly stimulate IEC PFKFB3 expression.

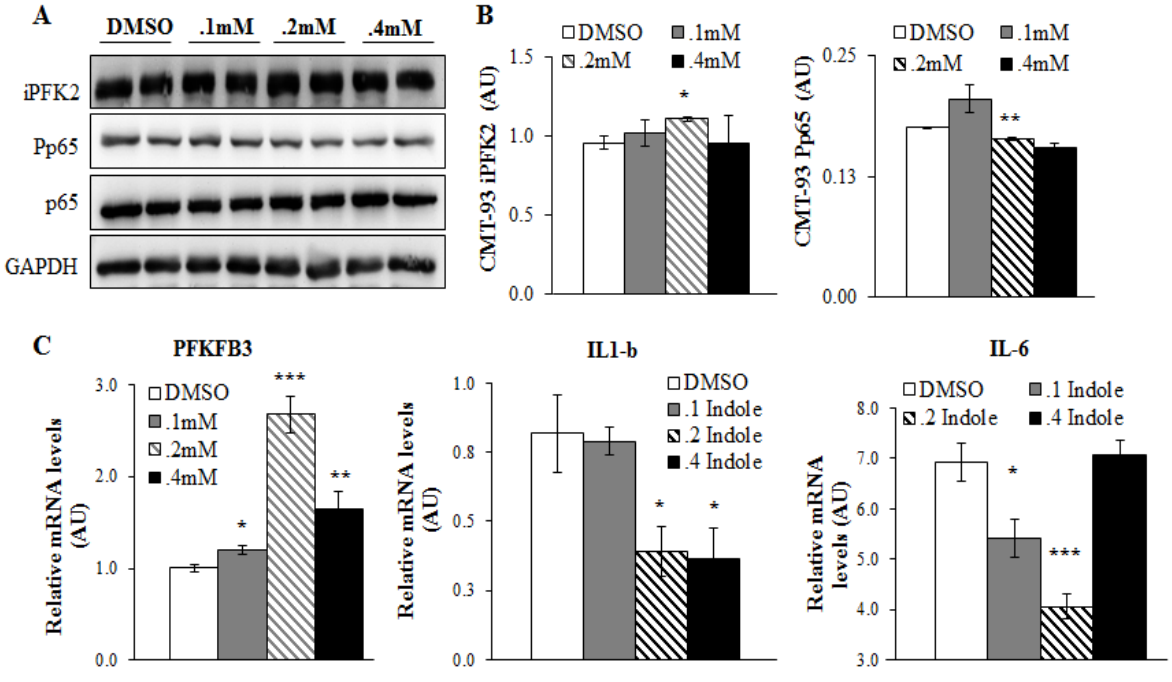

Figure 17 Dose effect of indole on IEC iPFK2 and proinflammatory responses. CMT-93 cells were treated as described in methods. (**A**) Western blot analyses of IEC iPFK2 amount and NF-κβ signaling. (**B**) Quantification of IEC iPFK2 and Pp65/p65. (**C**) IEC expression of PFKFB3, IL1-β and IL-6 mRNAs was determined using real-time PCR. iPFK2, inducible 6-phosphfructo-2-kinase; IL-1β, interleukin-1 beta; IL-6, interleukin-6. For A and B, CMT-93 cells were treated with .1mM, .2mM or .4mM indole or dimethyl sulfoxide (DMSO, control) for 24 hr. For B and C, data are means ± S.E. n = 4. *, $P < 0.05$ and **, $P < 0.01$, ***, $P < 0.001$ indole concentration vs DMSO for the same gene.

HFD-induced insulin resistance and glucose intolerance

To determine any value of indole for treatment of obesity-associated inflammation and insulin resistance, the present study dosed HFD-fed mice with PBS or indole daily via

oral gavage for 6 weeks. PBS- and indole-dosed mice displayed similar body weights throughout the experiment and consumed comparable amounts of food (Fig. 19, A and B). While all mice developed obesity-related insulin resistance and glucose intolerance, indole-dosed mice displayed significantly lower plasma glucose levels as compared to the PBS-dosed group in the insulin tolerance test. (Fig.19C). Specifically, plasma glucose at 30, 45 and 60 min after the bolus insulin injection were all significantly lower than PBS-dosed mice, and the area-under-the-curve (AUC) calculation confirmed an overall significance. Two time points within the glucose tolerance test were significant but the AUC was similar between groups (Fig. 19D). To our knowledge this provides the first evidence of an insulin-sensitizing effect of indole and suggests that indole may be beneficial in combating hyperglycemia.

Stimulation of PFKFB3 in primary IECs

Data presented earlier provides evidence of how macronutrients regulate PFKFB3 expression in primary IECs; however, the influence of other factors, namely metabolites, is unknown. The present study therefore investigated how oral administration of indole affects PFKFB3/ iPFK2 *in vivo*. Western blot images suggest a slightly increased iPFK2 amount in HFD indole-dosed mice, compared to HFD+PBS mice (Fig. 20A). Unfortunately, due to difficulties in isolating adequate amounts of IEC protein, western blot results could not be quantified. However, given that indole dosage *in vivo* is relatively novel, as well as the interaction between indole and PFKFB3/iPFK2, it is important to present these images nonetheless as representative data.

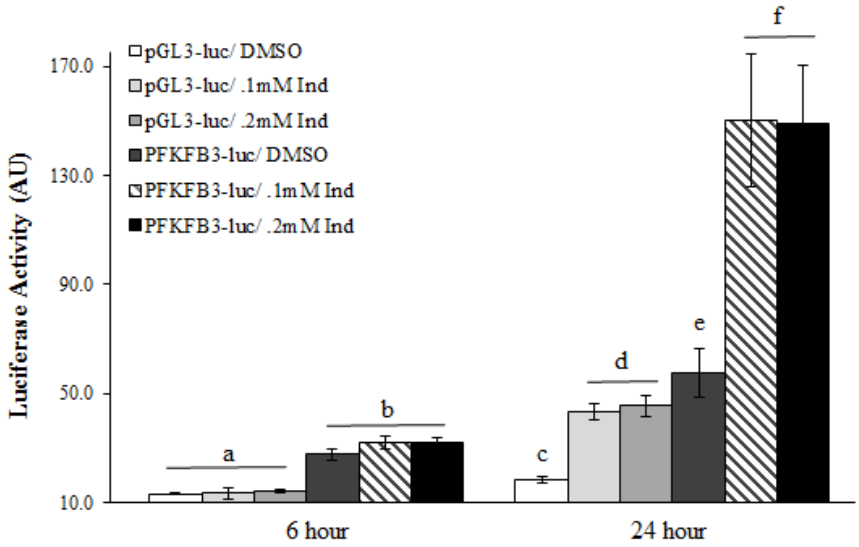

Figure 18 Indole stimulates PFKFB3 gene transcription. CMT-93 cells were transfected with pGL3-PFKFB3-luc or a control reporter construct (pGL3-luc) and exposed to .1mM or .2mM indole or DMSO (control) for an additional 6 hr or 24 hr. Data are means ± S.E. n = 6. *, $P < 0.05$ indole vs DMSO for the same treatment (pGL3-PFKFB3-luc/indole or pGL3-PFKFB3-luc/DMSO).

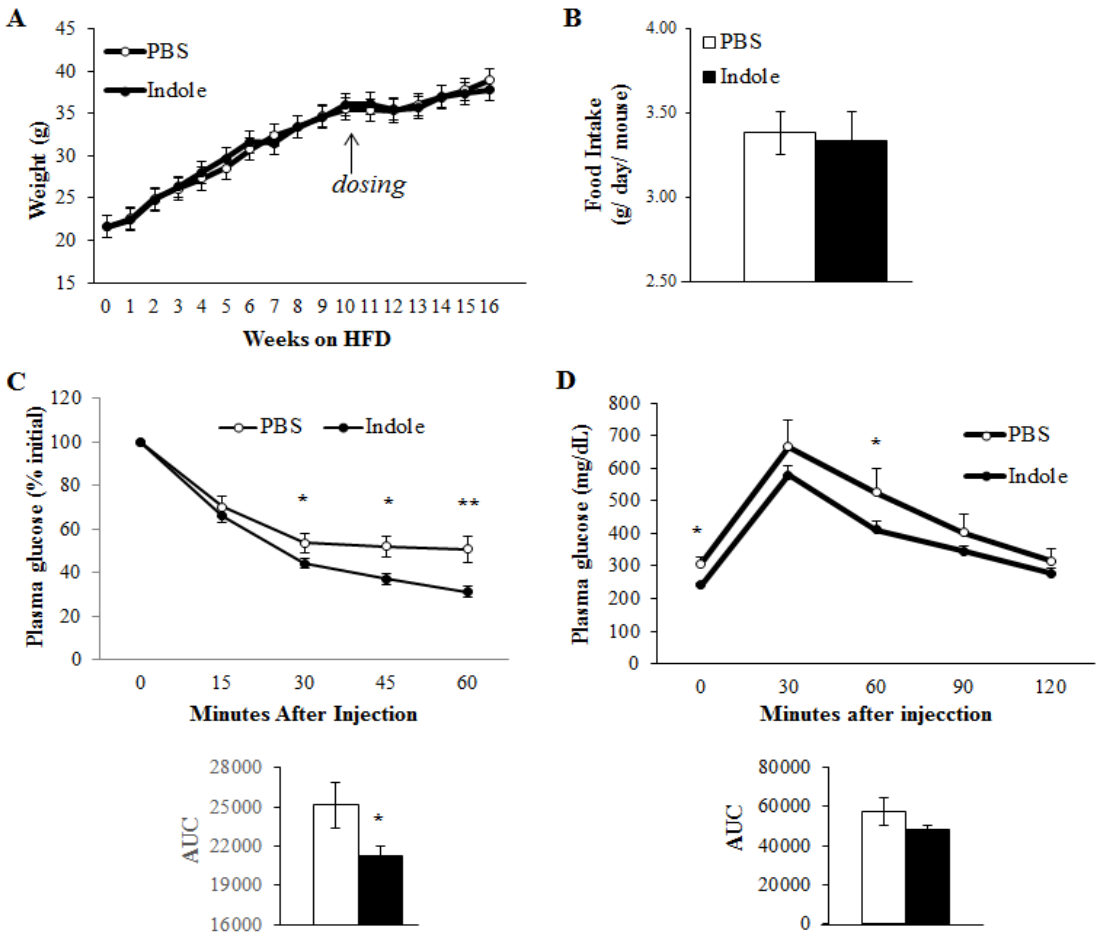

Figure 19 Indole effects on HFD induction of obesity-related insulin resistance and glucose intolerance. Male C57BL/6J mice, at 5 – 6 weeks of age, were fed a high-fat diet (HFD) for 16 weeks. n = 20. Mice were divided randomly and dosed daily via oral gavage with indole (50mg/kg, HFD-Indole) or PBS (control, HFD-PBS). (**A**) Body weight. (**B**) Food intake. (**C**) Insulin tolerance tests. After the feeding regimen, the mice were given a bolus intraperitoneal injection of insulin (1 U/kg). (**D**) Glucose tolerance tests. For C and D, areas under curves (AUC) were calculated based on the corresponding tolerance tests. For A – D, data are means ± SE. *, $P < 0.05$ and ** $P < 0.01$ HFD-PBS vs HFD-Indole (B; AUC in C and D) for the same time point (A, C, and D).

mRNA results do support western blot images however, as they show a significantly increased PFKFB3 amount in the HFD, indole-dosed group compared to that of HFD-PBS mice (Fig. 20B). Therefore, it appears indole can possibly prevent the loss of PFKFB3 caused by HFD feeding. HFD-induced loss of PFKFB3 expression has been shown previously and is further confirmed by both western blot and rtPCR results presented here.

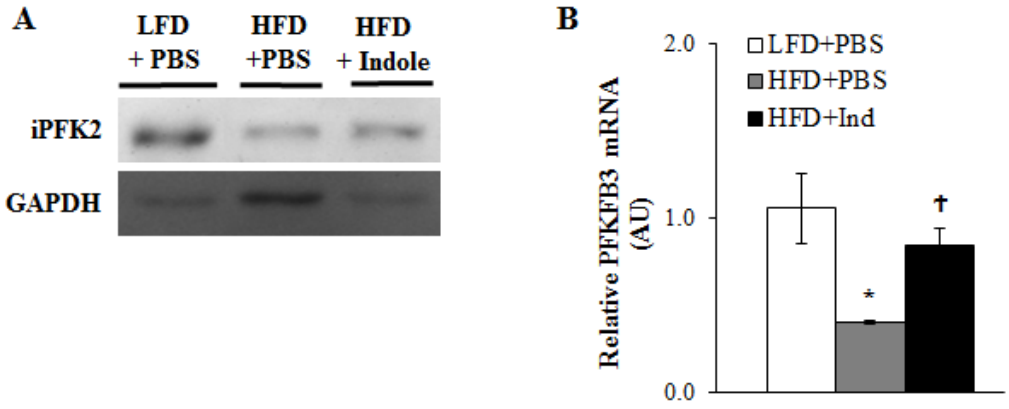

Figure 20 Oral indole increases PFKFB3/iPFK2 amount *in vivo*. Primary IECs were isolated as described in methods. IECs were isolated from LFD- or HFD-fed mice dosed with indole or PBS (control). Data are means ± S.E. n = 5. *, $P < 0.05$ HFD vs LFD for same treatment (PBS); †, $P < 0.05$ indole vs PBS within the same diet (HFD).

DISCUSSION

This study provides evidence for the first time of an interaction between the bacterial metabolite indole and PFKFB3/iPFK2. Specifically, indole exposure *in vitro* is associated with increased PFKFB3/iPFK2 expression and reduced levels of inflammatory markers in CMT-93 cells. A direct influence of indole on PFKFB3/iPFK2 was confirmed

by gene transcription reporter assay. In support of *in vitro* experiments, i*n vivo* data show that HFD-fed, indole-dosed mice possibly exhibit higher protein and mRNA levels of PFKFB3/iPFK2 compared to HFD-PBS mice. Further, results from glucose and insulin tolerance tests indicate an insulin-sensitizing action of indole.

Results from *in vitro* experiments indicate significant increases in both PFKFB3 mRNA and iPFK2 protein levels following exposure to 0.2 mM indole. This finding is significant as it further elucidates factors that play a role in the regulation of PFKFB3, a primary stimulatory gene of glycolysis. While this is the first study to investigate the association between these molecules, previous studies have shown that indole positively influences a number of other genes and hormones involved in metabolism. For instance, indole stimulates glucagon-like peptide-1 (GLP-1) release from intestinal L cells (156), which in turn drives the release of insulin from the pancreas. Further, indole contributes to the generation of phosphoinositide-3-kinase (PI3K) and Akt (154) which, in addition to being upstream of the anti-inflammatory molecule IL-10, play important roles in maintaining normal insulin signaling. Thus, indole appears to positively contribute to insulin signaling in a variety of ways. The present study further sheds light on the impact of indole on a primary gene involved in another metabolic pathway, glycolysis.

The presence of indole also significantly reduced the protein levels of two proinflammatory markers, NF-κB and TLR4. NF-κB, a transcription factor phosphorylated by numerous stimuli including growth factors, LPS, IL-6, and TNFα (157,158), is known to be a major stimulant in the inflammatory response within intestine (159,160). TLR4, one of the prominent toll-like receptor isoforms localized in intestine,

is a well-known proinflammatory marker as well. TLR4 not only regulates the production of inflammatory cytokines in response to stress stimuli such as LPS (161), but also following overnutrition with saturated fat (88). Thus, the anti-inflammatory action of indole within intestinal cells seen in the present study appears to be twofold, as it is associated with reduced levels of both major inflammatory markers. In particular, indole's ability to reduce TLR4 expression is especially exciting as it indicates its potential in combating overnutrition-associated inflammation. While previous reports have shown anti-inflammatory abilities of indole (154), they did not investigate any relationship between indole and TLR4. Thus, the present study not only confirms the actions of indole on inhibiting NF-κB signaling in intestine, but provides the first account for its specific anti-inflammatory action on TLR4 within IECs.

The present study also tested varying concentrations of indole (0.1 mM and 0.4 mM) to determine any dose-effect on either PFKFB3/iPFK2 or inflammatory cytokine levels. However, results from both protein and mRNA analyses indicate less PFKFB3/iPFK2 at all other doses compared to 0.2 mM indole. In addition, expressions of all inflammatory markers, p-NF-κB, IL-1β, and IL-6, were the lowest at 0.2 mM indole. Together these findings suggest that 0.2 mM indole may be the most beneficial concentration for the balance between increased PFKFB3/iPFK2 and reduced inflammatory markers within IECs. Previous studies have used significantly higher concentrations (1 mM) of indole in testing its anti-inflammatory ability (154). While this approach is valid given that human intestinal cells may be exposed to indole levels up to 600 μM (162,163), this concentration is not feasible for the *in vitro* experiments conducted

in the present study. Firstly, as a developed, stable cell line, CMT-93 cells have been separated from the intestinal epithelium for a significant amount of time and therefore are not exposed to many intrinsic factors that normally occur within the intestinal environment. Secondly, the high indole concentrations seen within the intestinal environment (600μM) applies primarily to humans. Thus, experimental concentrations used previously in human cells (1 mM) may be toxic to murine cell lines. Indeed, the present study did attempt higher concentrations (0.25 mM, 0.5 mM, and 1 mM) of indole in initial *in vitro* experiments. However, significant cell death was observed at all concentrations other than 0.25 mM (data not shown). Therefore, the experiments were repeated using lower concentrations to better address the major questions posed in this study. Although the concentrations in this study are lower than those used previously, the results presented here are significant nonetheless as the CMT-93 cell line remains a useful tool given that cells exhibit similar morphology, and metabolic and inflammatory signaling pathways as primary IECs.

The positive association between indole and PFKFB3/iPFK2 expression led the present study to gain insight into how indole exerts this effect. Results from the luciferase reporter assay indicate a direct relationship between indole and PFKFB3. Specifically, data show that 24 hr exposure to either 0.1 mM or 0.2 mM indole significantly increases PFKFB3 promoter activity compared to the DMSO control. This same finding was not present after 6 hr exposure to treatments. Therefore, longer exposure to indole seems most beneficial for the stimulation of PFKFB3 expression. 24 hr was the longest treatment included in this study but prolonged experiments are necessary to determine if this result

remains, especially given that IECs can be continuously exposed to bacterial metabolites such as indole.

Given that inflammation is a major underlying factor in the generation of overnutrition-induced obesity and metabolic disease (56,111), and that indole exerts anti-inflammatory actions both in the intestinal environment and specifically within IECs, the present study investigated whether indole is beneficial in the treatment of HFD-induced obesity and insulin resistance. Glucose tolerance tests only showed significant differences in plasma glucose level at initial and 60' time points, with no difference between groups in the AUC calculation. Interestingly however, indole dosing resulted in significantly lower plasma glucose levels in the insulin tolerance tests. Specifically, all time points 30 min and later were significant, as well as an overall significance, as indicated by AUC calculations. This finding is exciting as it suggests a possible systemic insulin-sensitizing effect of indole which has not been shown previously. Many studies have implicated diet-induced inflammation generated within the intestine in the onset of inflammation in distal tissues (100,164). In fact, intestinal inflammation is known to precede the onset of obesity and systemic insulin resistance (99). Therefore, indole may be beneficial in not only reducing the intestinal inflammatory response to overnutrition, but may help lessen the subsequent metabolic dysfunction commonly seen in distal tissues. This is the first study to our knowledge investigating oral administration of indole for treatment of obesity and insulin resistance. Therefore, more research is needed to fully explore both the intestinal and systemic effects of indole to determine its full potential as a therapeutic target.

Oral administration of indole was also associated with slightly increased PFKFB3/iPFK2 protein and mRNA levels, as compared to HFD-PBS dosed mice. Although western blot images could not be quantified due to low protein amount, and therefore only serve as representative data, these trends, especially combined with mRNA results, support *in vitro* findings in that indole stimulates PFKFB3 expression. Further, PFKFB3 mRNA in HFD-Indole mice was actually comparable to that of LFD-PBS animals so it appears that indole not only prevents the loss of PFKFB3 mRNA caused by HFD-feeding, but can stimulate its expression to levels found in LFD-fed animals. However, further study is needed to validate this view.

In summary, indole stimulates PFKFB3/iPFK2 expression and regulates the generation of pro-inflammatory cytokines within IECs. Results presented here not only add to the current knowledge regarding beneficial abilities of indole within the gut environment, but also specifically within epithelial cells, where nutrients first interact with host cells. Whether indole is indeed beneficial for the treatment of HFD-induced obesity and metabolic diseases such as insulin resistance remains to be confirmed. Nonetheless, indole is continuing to prove an exceedingly beneficial molecule within the intestine.

CHAPTER VI

SUMMARY

Obesity, and a number of associated metabolic disorders, are major health concerns for many adult Americans. Obesity-associated inflammation appears to be one of the major contributing factors to the generation of such disorders, so research to investigate the underlying mechanisms of inflammation and how it interferes with normal metabolism is vital. Previous studies have primarily concentrated on metabolic and inflammatory pathways in adipose tissue, with few focusing on the small intestine, where nutrients first interact with the body. PFKFB3/iPFK2, an essential metabolic enzyme involved in the regulation of glycolysis, has been shown to suppress inflammatory responses in adipose tissue; however, its role in the intestine is largely unknown. Therefore, the present study first investigated how diet/overnutrition and individual macronutrients influence PFKFB3/iPFK2 expression in intestinal epithelial cells (IECs), and second, determined how altered PFKFB3/iPFK2 relates to the generation of inflammation.

Similar to previous findings, overnutrition with saturated fat was associated with increased inflammatory responses but reduced expression of PFKFB3/iPFK2 in IECs. These results appear to be regulated by macronutrients which differentially influence metabolic and inflammatory signaling pathways. Specifically, carbohydrates (e.g. glucose) stimulate PFKFB3/iPFK2 expression whereas saturated fats, namely palmitate,

contribute to the generation of inflammatory responses. Further, PFKFB3/iPFK2 expression was inversely associated with the levels of inflammatory responses. Therefore, high intake of saturated fat versus carbohydrates contribute to increased inflammatory responses in small intestine and appear to interfere with normal stimulation of genes involved in metabolism (e.g. PFKFB3/iPFK2).

In line with these findings, disruption of PFKFB3/iPFK2 combined with overnutrition lead to significant inflammatory responses in IECs. Further, systemic insulin sensitivity and glucose tolerance was worsened with gene disruption, suggesting a role for PFKFB3/iPFK2 in overall glucose metabolism. The latter confirmed the findings of previous studies. On the other hand, PFKFB3/iPFK2 overexpression in intestinal cells lead to reduced inflammatory responses while inhibition of PFKFB3/iPFK2 was associated with increased inflammation. Thus, PFKFB3/iPFK2 in small intestine has anti-inflammatory abilities similar to those seen in adipose tissue.

Due to the fact that many dietary, metabolic, and bacterial factors converge within the small intestine, it is likely that factors other than nutrients also influence IEC PFKFB3/iPFK2 expression. Indeed, the present study provides evidence that bacterial metabolites may have such an ability. Indole, a metabolite produced from bacterial digestion of tryptophan, stimulates PFKFB3/iPFK2 expression and reduces the generation of inflammation. Indole has known anti-inflammatory abilities within IECs but the extent of which are not fully understood. Therefore, an additional mechanism of action appears to be through PFKFB3/iPFK2. Indole also appears to play a beneficial role in regulating overnutrition-induced hyperglycemia.

In conclusion, these findings together suggest an anti-inflammatory role of PFKFB3/iPFK2 in small intestine. Given its regulation by numerous factors, namely macronutrients, PFKFB3/iPFK2 appears to play a vital role in balancing metabolic and inflammatory signaling pathways within IECs, thereby contributing to the regulation of systemic insulin sensitivity and energy homeostasis.

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