

**BENEFICIAL EFFECTS OF METFORMIN IN DIET-INDUCED OBESITY
ASSOCIATED NON-ALCOHOLIC FATTY LIVER DISEASE**

A Dissertation

by

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is closely associated with obesity and insulin resistance. To better understand the pathophysiology of obesity-associated NAFLD, the present study examined the involvement of liver and adipose tissues in metformin actions on reducing hepatic steatosis and inflammation during obesity. Also, the importance of AMPK in regulating the anti-steatosis and anti-inflammatory properties of metformin was examined.

C57BL/6J mice were fed a high-fat diet (HFD) for 12 weeks to induce obesity-associated NAFLD and treated with metformin (150 mg/kg/d) orally for the last four weeks of HFD feeding. Compared with HFD-fed control mice, metformin-treated mice showed improvement in both glucose tolerance and insulin sensitivity but also a decrease in hepatic steatosis and liver inflammation associated with an increase in the phosphorylation of liver AMP-activated protein kinase (AMPK). However, metformin treatment did not significantly alter adipose tissue AMPK phosphorylation and inflammatory responses. *In vitro* studies showed that metformin directly decreased steatosis in hepatocytes and inflammation in both hepatocytes and macrophages, and is AMPK associated. Further *in vitro* studies confirmed the importance of AMPK in regulating the anti-steatosis and anti-inflammatory effects of metformin in hepatocytes and macrophages.

Taken together, these results suggest that metformin protects against obesity-associated NAFLD largely through direct effects on decreasing hepatocyte fat deposition and on inhibiting inflammatory responses in both hepatocytes and macrophages.

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NOMENCLATURE

AMPK	5' Adenosine Monophosphate-activated Protein Kinase
ACC	Acetyl-CoA Carboxylase
AP-1	Activator Protein 1
BMDM	Bone Marrow-Derived Macrophage
ChREBP	Carbohydrate Response Element-Binding Protein
CC	Compound C
CPT1	Carnitine Palmitoyltransferase I
DAMPS	Damage-Associated Molecular Patterns
DNL	De Novo Lipogenesis
FFAs	Free Fatty Acids
FAS	Fatty Acyl-CoA Synthase
FOXO1	Forkhead Box O1
GLUT4	Glucose Transporter 4
GTT	Glucose Tolerance Test
HFD	High Fat Diet
HGP	Hepatic Glucose Production
IKK	I κ B Kinase
IL-1 β	Interleukin-1 β
IL-4	Interleukin-4
IL-6	Interleukin-6
IR	Insulin Resistance

ITT	Insulin Tolerance Test
JNK	c-Jun N-terminal Kinase
LXR	Liver X Receptor
LPS	Lipopolysacharride
MCD	Methionine- and Choline-Deficient
MCP-1	Monocyte Chemotactic Protein-1
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NAFLD	Nonalcoholic Fatty Liver Disease
NASH	Nonalcoholic Steatohepatitis
NF κ B	Nuclear Factor κ B
PAMPS	Pathogen-Associated Molecular Patterns
PGC1- α	PPAR Gamma Co-activator 1- α
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RXR	Retinoid X Receptor
SREBP-1c	Sterol Regulatory Element-Binding Protein 1c
SVC	Stromal Vascular Cell
T2DM	Type 2 Diabetes Mellitus
TG	Triglycerides
TLR-4	Toll-Like Receptor-4
TNF- α	Tumor Necrosis Factor- α
VLDL	Very Low Density Lipoprotein

WAT White Adipose Tissue

WT Wild-Type

TABLE OF CONTENTS

	Page
ABSTRACT	ii
ACKNOWLEDGEMENTS	iii
NOMENCLATURE	iv
TABLE OF CONTENTS	vii
LIST OF FIGURES	ix
CHAPTER I INTRODUCTION	1
CHAPTER II LITERATURE REVIEW	3
Non-alcoholic disease, obesity, and type 2 diabetes.....	3
Nutrition regulation of lipogenesis	6
Obese adipose tissue and NAFLD	11
Hepatic insulin resistance and NAFLD	14
Macrophage involvement in NAFLD	16
AMPK: a pleiotropic kinase that regulates metabolism and inflammation	18
Metformin action in the liver	20
Summary	23
CHAPTER III METFORMIN AMELIORATES HEPATIC STEATOSIS AND LIVER INFLAMMATION IN VIVO	24
Introduction.....	24
Materials and methods	26
Results.....	30
Discussion.....	38
CHAPTER IV METFORMIN DOES NOT IMPROVE ADIPOSE TISSUE METABOLIC PHENOTYPE AND INFLAMMATORY STATUS	40
Introduction.....	40
Materials and methods	41
Results.....	44
Discussion.....	49

CHAPTER V METFORMIN HAS A DIRECT EFFECT ON HEPATOCYTES AND MACROPHAGES IN IMPROVING LIPID METABOLISM AND LIVER INFLAMMATION	50
Introduction.....	50
Materials and methods.....	51
Results.....	54
Discussion.....	60
CHAPTER VI METFORMIN ACTION ON IMPROVING NAFLD INVOLVES ACTIVATION OF AMPK IN HEPATOCYTES AND/OR MACROPHAGES	62
Introduction.....	62
Materials and methods.....	64
Results.....	66
Discussion.....	71
CHAPTER VII SUMMARY AND CONCLUSION	74
Summary.....	74
Conclusions.....	77
REFERENCES	78

LIST OF FIGURES

	Page
Figure 1. Metformin treatment ameliorates HFD-induced insulin resistance and glucose intolerance.	31
Figure 2. Metformin treatment ameliorates HFD-induced hepatic steatosis.....	34
Figure 3. Metformin treatment increases liver AMPK phosphorylation in HFD-fed mice.....	35
Figure 4. Metformin treatment ameliorates HFD-induced liver inflammatory responses	37
Figure 5. Metformin treatment does not alter HFD-induced adiposity.....	45
Figure 6. Metformin treatment does not alter HFD-induced adipose tissue inflammation	47
Figure 7. Metformin treatment does not alter adipose tissue AMPK status and inflammation.	48
Figure 8. Metformin treatment blunts hepatocyte fat deposition.	55
Figure 9. Metformin treatment increases AMPK phosphorylation, and decreases inflammatory responses in hepatocytes.....	57
Figure 10. Metformin treatment suppresses macrophage pro-inflammatory activation	59
Figure 11. Treatment of CC blunts the anti-steatosis effect of metformin in primary mouse hepatocytes	69
Figure 12. The confirmation of $\alpha 1$ AMPK $^{-/-}$ BMDM.	70
Figure 13. Metformin treatment has no effect on decreasing inflammatory signaling in WT hepatocytes / $\alpha 1$ AMPK $^{-/-}$ macrophages co-culture.	70

CHAPTER I

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of liver diseases that is increasingly shown to be associated with elevated risk of complications and liver-specific mortality (1). At present, there has yet to be a standard treatment for managing NAFLD. Since the occurrence of NAFLD is highly prevalent in obese and type 2 diabetic individuals (2,3), suggested approaches in managing patients with NAFLD and concurrent diabetes aim at improving glucose metabolism, which include metformin treatment.

Metformin, a drug that is widely used in controlling type 2 diabetes mellitus (T2DM), has been increasingly shown to be beneficial in improving NAFLD. The mechanism of metformin is mainly considered to be closely associated with AMP-activated protein kinase (AMPK), an energy-sensing molecule that regulates cellular and systemic energy homeostasis. Metformin not only is able to decrease hepatic gluconeogenesis, but also, in many studies, has been shown to decrease hepatic steatosis (4,5). But, whether or not metformin decreases hepatic inflammatory damage, histological features of NASH remain controversial. Although there are studies supporting the effect of metformin in decreasing hepatic inflammation (6,7), the exact cellular mechanisms as to how metformin decreases liver inflammation remains unclear. Knowing that liver inflammation is important in the progression of NAFLD, we would like to focus on exploring the cellular mechanisms of how metformin decreases liver inflammation. Therefore, the main hypothesis of this dissertation is that metformin could

ameliorate hepatic steatosis and liver inflammation through AMPK activation, directly affecting hepatocytes and macrophages, and indirectly improving adipose tissue phenotype in an obesity-related NAFLD mice model.

CHAPTER II

LITERATURE REVIEW*

NON-ALCOHOLIC FATTY LIVER DISEASE, OBESITY, AND TYPE 2 DIABETES

Non-alcoholic fatty liver disease (NAFLD) is a clinical condition that encompasses the whole spectrum of liver diseases including hepatic steatosis, non-alcoholic steatohepatitis (NASH), and cirrhosis without significant alcohol consumption. While simple lipid accumulation in the liver is generally considered as histologically benign, it could progress to NASH during overt liver necroinflammation. Moreover, the risk of liver progression from benign to NASH increases as lipid accumulation of more than 5% the liver volume or weight, or hepatocytes containing more than 5% visible intracellular triglycerides, by means of either imaging or biopsy (8). Chronic progression of NASH could potentially progress to cirrhosis, liver failure and liver cancer. Therefore, NASH is considered as the more progressive and damaging form of NAFLD. It has been estimated that the prevalence of NAFLD ranges from 6% to 35% with a median of 20% worldwide in the general population (2,9). Among the biopsied NAFLD patients, it is estimated that the prevalence of NASH is approximately

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30% (10). In addition, NAFLD is the most common liver disease in industrialized Western countries. In fact, NASH is becoming a more common cause for liver transplantation in the United States, and is on the path of becoming the most common (11,12).

NAFLD is increasingly observed to coexist with obesity and insulin resistance, which are components of metabolic syndrome that increases the risk of cardiovascular disease (13). The close relationship between obesity, insulin resistance, and NAFLD came from the observations in fatty changes in liver of obese patients, and in the presence of hyperinsulinemia and glucose intolerance in obese patients (14-16). In fact, recent studies estimated that the prevalence of NAFLD among obese patients is 60% (10), and that the prevalence of NAFLD among diabetes patients is 50% (17).

Although the pathogenesis of NAFLD is not fully understood, NAFLD could be represented by a “two hits” model that was first proposed by Day and James (18). The first “hit” requires the production of hepatic steatosis. Factors that contribute to hepatic steatosis include increased hepatic *de novo* lipogenesis, decreased hepatic β -oxidation, increased free fatty acid supply from adipose, and decreased very-low density lipoprotein (VLDL) triglyceride output (19-21). The second “hit” requires a source of oxidative stress capable of initiating significant lipid peroxidation, leading to histological damage (18); though nowadays, there is more and more evidence showing that the second “hit” could be promoted by a chronic proinflammatory environment induced by obesity-related adipose tissue dysfunction and obesity-induced insulin resistance. This is important, as adipose dysfunction is a critical source of adipocytokines such as

interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) that could promote liver inflammation (NASH) (22). However, the sequence of these “two hits” has been challenged in the sense that inflammation could precede hepatic steatosis and the metabolic events present in NAFLD are suggested to occur in a parallel rather than a consecutive manner. In addition, emerging evidence suggests that there are multiple factors contributing to NAFLD concurrently. These factors that lead to liver inflammation include gut-derived mediators, adipose-derived mediators, and endoplasmic reticulum stress. Therefore, a “multiple parallel hits” concept might be a more precise reflection of the current conception of NAFLD pathology (19).

Studies in rodents and humans have revealed that the excessive accumulation of triglyceride in the liver is mainly caused by an overflow of fatty acids from hypertrophied and insulin resistant adipose tissue, and from increased de novo lipogenesis (DNL) in the liver; but is less contributed by fatty acid disposal through β -oxidation or VLDL export (23). In fact, isotopic quantitative analysis in NAFLD patients showed that labelled triglyceride in the liver was distributed around 59% derived mainly from adipose lipolysis, 26% derived from DNL, and 15% derived from the diet (24). Importantly, the lipogenic rate, which is normally inhibited during fasted state, was already elevated during.

NUTRITION REGULATION OF LIPOGENESIS

Since the liver is the major site of de novo lipogenesis (DNL) in humans (25), it is worth mentioning the effects of nutrients and how they affect hepatic lipogenesis when in excess. Hepatic DNL is generally most activated by high-carbohydrate feeding but inhibited by high-fat feeding and fasting. Overfeeding of carbohydrate enhances the conversion of carbohydrate into fatty acids. Perhaps one very good example of this phenomenon is the clinical observation of fatty infiltration in liver induced by high dextrose content of total parenteral nutrition (26).

Numerous studies have also shown the lipogenic effect of carbohydrate overfeeding (27,28). Particularly, during massive overfeeding of a carbohydrate-rich diet in the Guru Walla model – a Cameroon ritual where teenage boys deliberately overconsume a 7000 kcal/day, high carbohydrate diet (70%) but just a total of 4 kg fat consumption in over 10 weeks, which they gain 12 kg of fat (29). However, during less extreme carbohydrate feeding, DNL is less significant. As shown by Acheson et al., DNL becomes significant after maximum whole-body glycogen storage of approximately 700-1000 g is reached, which equates to averagely 800 g/day of carbohydrate for 4 days as shown in his study. In other words, the excess carbohydrate during short-term ingestion of a large amount of carbohydrate should go towards saturation of glycogen storage before DNL becomes significant – net lipid synthesis exceeds glycogen storage, and carbohydrate and lipid oxidation (30,31).

While the above given examples did not explain the lipogenic effect related to the composition of carbohydrate, it is generally recognized that glucose is the main

lipogenic substrate in monogastric animals. Fructose is also now emerging as a strong lipogenic stimulator. Although fat is generally thought to antagonize lipogenesis, there is accumulating evidence that different fatty acids will have different effects on lipogenesis. And, these will be discussed further in the sections below.

Glucose

The role for glucose in stimulating DNL has long been recognized. This is because glucose, through its metabolism, provides substrates that are needed for DNL. Furthermore, as a signaling molecule, glucose has a stimulatory effect on the expression of several key lipogenic enzymes, in particular acetyl-CoA carboxylase (ACC) (32,33). However, this role should be interpreted as a role for glucose metabolism, but not glucose, *per se*, in stimulating DNL. Physiologically, glucose metabolism is tightly coupled to insulin. Thus, it is very difficult to separate the effect of glucose from that of insulin on DNL. Since the identification and functional characterization of carbohydrate response element-binding protein (ChREBP) in controlling the expression of lipogenic enzymes (34), it became largely accepted that ChREBP and sterol regulatory element-binding protein-1c (SREBP-1c) mediate the effect of glucose and insulin, respectively, on stimulating lipogenic enzyme expression.

The stimulatory effect of glucose, strictly speaking glucose metabolism, on DNL is manifested by the finding that glucokinase overexpression in rodents led to hepatic steatosis (35,36). A simple explanation is that enhancing glycolysis provides substrates for DNL. Because generation of fructose-1,6-bisphosphate from fructose-6-phosphate is

a rate-determining step of glycolysis, overexpression of a kinase-dominant 6-phosphofructo-2-kinase was used to enhance glycolysis (36). Of interest, DNL was not increased; although glycolysis was enhanced. Further mechanistic investigation demonstrated the importance of pentose phosphate shunt in activating ChREBP, and, likely, in generating NADPH, another required substrate of DNL, to enable DNL (36). In that study, insulin levels were decreased in response to glucokinase overexpression. This, however, does not imply a stimulatory effect of glucose (metabolism) on DNL that is independent of insulin. Indeed, enhancing glycolysis through overexpression of glucokinase generates effects on glycolysis that are almost identical to the combined effect of glucose and insulin.

Fructose

Fructose has received much attention recently due to its association with extensive dietary consumption in the modern society, and its close relationship with metabolic diseases such as NAFLD, insulin resistance, and dyslipidemia, due mostly to the lipogenic capacity of fructose.

Fructose is a monosaccharide (as are glucose and galactose) that occur in the form of sucrose, a disaccharide composed of fructose and glucose, or in the form fructose alone. After absorption, fructose is rapidly phosphorylated by fructokinase into fructose-1-phosphate in hepatocytes. Fructose-1-phosphate is then converted to glyceraldehyde and dihydroxyacetone, which could feed into the regular glycolytic pathway. This is of importance because the fructose metabolic pathway is able to bypass

the main regulatory step of glycolysis, which is 6PFK1. In addition, fructose uptake is not regulated by insulin nor does fructose stimulate insulin secretion as much as glucose does, and fructokinase has almost 10 times higher enzyme activity than glucokinase (37). Moreover, Havel et al. showed that an equivalent dose of glucose and fructose (1 g/kg dose) produced dissimilarities in their levels reflected in blood. Blood fructose level increases minimally to approximately 0.5mM, which is relatively low when compared to 10mM postprandial blood glucose level (38). This suggests that fructose is highly metabolized upon the first pass to the liver.

There is no doubt that fructose stimulates the mRNA expression of lipogenic enzymes such as ACC and FAS (39). However, this does not mean that all the fructose is metabolized in favor of lipogenesis. In fact, in a single-blinded, randomized, crossover study, it was found that 35% of the fructose was oxidized, approximately 38% appeared to form glycerol in the form of VLDL-triacylglycerol, 0.4% appeared as de novo fatty acids in the form of VLDL-triglyceride, and some likely remained storage as liver glycogen after a fructose load of 0.75 g/kg (40).

The contemporary issue is that the over-consumption of sucrose and high-fructose corn syrup in our daily food products likely is amplifying the significance of the lipogenic effect of fructose (41). Moreover, fructose could induce hepatic and extrahepatic insulin resistance as shown by Dirlewanger et al. (42). Also, high fructose consumption has been highly correlated with visceral obesity (43,44). This is important as insulin resistance and obesity are highly related to metabolic diseases such as NAFLD and dyslipidemia (45-47), which will be discussed further.

Fatty acid composition

The earliest report of the inhibitory effect of dietary lipid on hepatic lipogenesis is in 1950 when Masoro et al. showed that there were less labelled [^{14}C] fatty acids generated from [^{14}C] glucose in rat livers after 3 days of a diet containing 60% butter, compared to rats that were fed a 60% glucose diet (48). Due to the heterogeneity of dietary lipid composition, such as saturated, mono-, and poly-unsaturated fatty acids, numerous subsequent studies then aim to clarify if all dietary fats have equal effects on diminishing DNL. Saturated fatty acids promotes fatty acid synthesis (49), whereas polyunsaturated fatty acids (PUFA), both n-3 and n-6 PUFAs, are able to suppress the hepatic lipogenic enzymes (50-53). Interestingly, palmitoleate, a monounsaturated fatty acid, seems to controversially stimulate DNL as observed in the inducing effect of palmitoleate in increasing FAS mRNA expression in mice liver, as well as an increase in hepatic lipid accumulation (54).

Palmitoleate

As mentioned above, palmitoleate controversially increases DNL. In two lines of mice studies (55,56), palmitoleate was thought to decrease hepatic lipid accumulation due to inhibition of FAS expression in liver. In contrast, Petit et al. reported that circulating palmitoleate level might positively correlate with the degree of hepatic steatosis in human subjects (57). A recent mechanism study by Guo et al. reported similar effects of palmitoleate on inducing hepatic DNL and hepatic steatosis in mice (54). This is supported by an increase in expression of SREBP-1c and FAS upon

palmitoleate treatment in hepatocytes cell line. Interestingly, palmitoleate supplementation in mice also improved insulin sensitivity and glucose tolerance, thus dissociating the association between insulin resistance and hepatic steatosis. In particular, these effects are found to be activated via liver Akt signaling by palmitoleate (54), which is important as SREBP-1c is known to be activated via the Akt-mTORC1 pathway and gluconeogenesis is known to be downregulated via Akt-FOXO1 pathway. Also, the ability of palmitoleate to reduce liver and macrophage inflammatory response in this study might also help explain the improvement in insulin resistance (54).

The findings of this study provide a new insight of how different lipid composition could have profound effects on human physiology; as palmitoleic acid is a monounsaturated fatty acid that could be a dietary component and more importantly, could also be endogenously produced by adipocytes. The latter effect has important implication for further investigation on elucidating the cross-talk between adipose tissue and liver in the pathophysiology of metabolic diseases such as insulin resistance and NAFLD.

OBESE ADIPOSE TISSUE AND NAFLD

Adipose tissue not only functions as a mere energy/fat storage but is also a highly active endocrine organ that is made up of different cell populations including adipocytes, macrophages, and other immune cells. In an obese state, adipose tissue dysfunction can develop, resulting in lipid “overspill” and a chronic state of pro-inflammatory response, both of which could eventually lead to NAFLD.

Obesity and liver fat accumulation

Under normal conditions, circulating free fatty acids (FFAs) are contributed by adipose tissue lipolysis during post-absorptive conditions. These FFAs could enter the liver to be repackaged for fatty acid transport as VLDL. The major source of FFAs that enter the liver could come from subcutaneous adipose tissue, after passing through the splanchnic tissue and entering the hepatic artery and portal vein.

However, under obese condition, the proportion of FFAs coming from visceral adipose tissue entering the liver increases. It is of no surprise because visceral adipose tissue is known to be more pronounced, than other adipose tissues, to be drained by the portal circulation, which connects to the liver (58). Clearly, the major source of FFAs that enter the liver would come from increased visceral adipose lipolysis during post-absorptive and obese conditions (59). Indeed, as shown by a study that examined the relative FFAs in the portal vein originating from visceral adipose tissue of lean and obese subjects, it was found that while the hepatic FFA delivery from visceral adipose in lean subjects accounts for 5-10%, the proportion of hepatic FFA delivery from visceral adipose in obese subjects could be as high as 30% or more (60). This is important because visceral adipose tissue has been highly implicated to have a positive association with metabolic diseases, including NAFLD and insulin resistance.

Obesity-associated inflammation

During obese condition, the adipose tissue is undergoing many alterations including, adipocyte hypertrophy, nutrient overflow, increased antigens exposure from the gut, organelle stresses, all of which could directly or indirectly trigger the immune response in adipose tissue (61). In fact, adipose tissue inflammation, which is most represented by macrophage infiltration and increased inflammatory markers is positively associated with increased body fat mass/visceral fat, and adipocyte hypertrophy, and is a reversible process after weight loss (62,63). Similarly, adipose tissue macrophage infiltration is also greater in the adipose tissue of subjects with NAFLD (64).

In adipose tissue reside different cell populations including adipocytes and macrophages. In a condition of nutrient excess, as seen in obesity, these nutrients/metabolic signals could trigger pro-inflammatory intracellular pathways in adipocytes. The activation of the pro-inflammatory pathways, such as the c-Jun N-terminal kinases (JNK) and I κ B kinase (IKK) pathways could then induce a low-level production of cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), monocyte chemoattractant-1 (MCP-1) (65). Chronic activation of these inflammatory pathways and production of these cytokines may promote the recruitment and activation of immune cells, including macrophages, mast cells, T cells, etc. that in turn increases the production of those pro-inflammatory cytokines, driving the adipose tissue towards a state of low-grade inflammation. In effects, chronic state of low-grade inflammation induced by those cytokines could not only disrupt insulin signaling, resulting in insulin resistance, but also interrupt systemic metabolism. For

example, the discovery that TNF- α level was highly elevated in obese mice and that it could impair insulin signaling and glucose uptake in adipocytes have led to work in identifying the role of TNF- α in disrupting the insulin receptor signaling (66,67). Indeed, insulin resistance is important in linking obesity with metabolic diseases such as non-insulin dependent diabetes mellitus, NAFLD, and cardiovascular diseases.

In an obese adipose tissue, not only low-grade inflammation induces insulin resistance locally (i.e. adipose tissue), but the pro-inflammatory cytokines could also lead to insulin resistance in the peripheral tissues such as the liver (61).

HEPATIC INSULIN RESISTANCE AND NAFLD

Insulin resistance refers to insulin-responsive cells (such as hepatocytes, adipocytes, and myocytes) failed to respond to insulin signaling and the signal transduction is disrupted, therefore insulin-stimulated metabolism is impaired.

The liver represents an important metabolic organ that majorly participates in the metabolism of glucose and lipid – including gluconeogenesis and lipogenesis. The control of such metabolic pathways lies mainly in the ability of hepatocytes to respond properly to the signaling of insulin. Appropriate insulin signaling in the liver suppresses gluconeogenesis and promotes lipogenesis. However, during a state of hepatic insulin resistance, insulin signaling fails to suppress gluconeogenesis but insulin-induced lipogenesis remains functional. This “selective insulin resistance” paradox was proposed by Brown and Goldstein (68), and that impairment of insulin signaling diverges after protein kinase B (also known as Akt), suggesting that insulin-mediated suppression of

forkhead box protein O1 (FOXO1) is impaired while insulin-mediated sterol regulatory element-binding protein-1c (SREBP-1c) remains sensitive (69). Moreover, hyperinsulinemia that usually accompanies insulin resistance, as a compensation mechanism of the pancreas due to hyperglycemia, could further amplify the insulin-mediated lipogenesis. What causes the impaired suppression of gluconeogenesis but intact lipogenesis remains poorly understood. Accordingly, many theories have arisen to explain the pathology of this insulin signaling paradox (70-72). Of the very recent, there is evidence suggesting that over-nutrition-induced overproduction of triglyceride metabolites could result in hyper-activation of atypical protein kinase C (α PKC), and that this might be the key in selectively impairing FOXO1 phosphorylation while stimulation of SREBP-1c remains elevated (72). However, to be on the conservative side, more studies are still required to fully elucidate the molecular mechanisms of this selective insulin resistance.

It is important to recognize that there hasn't been a cause-and-effect relationship established between NAFLD and hepatic insulin resistance. This could be evidenced by the work of Samuel et al. (73). The authors showed that knocking down the expression of protein kinase C ϵ (PKC ϵ), which is known to impair insulin signaling, was able to reverse insulin resistance despite hepatic steatosis. They further showed that liver accumulation of diacylglycerol, which is a lipid metabolite, could activate PKC ϵ , and that PKC ϵ could directly bind and inhibit the activity of insulin receptor tyrosine kinase, thus impairing insulin signaling (73). In addition, hepatic steatosis is not always associated with insulin resistance, as evidenced by the work of Monetti et al., showing

that the hepatic steatosis induced by overexpression of diacylglycerol acyltransferase 2 (DGAT2), the committed step in triglyceride synthesis, did not result in insulin resistance (74). So, this begs the question: what accounts for the discrepancies of the existence between hepatic steatosis and insulin resistance? It has been suggested that when steatosis is associated with an accumulation of a specific lipid component, fatty acid, or lipid derivative, these component(s) could improve or impair insulin sensitivity (74). Indeed, a recent animal study by Huo et al. found an association between hepatic steatosis and an increase in palmitoleate level of the lipid content in the steatotic liver (75). To further verify the significance of this finding, Guo et al. demonstrated that palmitoleate was able to induce hepatic steatosis, by stimulating lipogenesis, but improve insulin sensitivity at both the hepatic and systemic level. More interestingly is that palmitoleate was able to suppress liver inflammation. This indicates that the improvement of insulin sensitivity by palmitoleate is, at least partly, due to the suppression of liver inflammation (54). This important finding also signifies that NAFLD is affected by nutrients themselves.

MACROPHAGE INVOLVEMENT IN NAFLD

The liver is a complex tissue composed of various cell population. Hepatocytes, which represents 60-80% of all liver cells, are responsible for most of the important metabolic and xenobiotic pathways of the liver. In the liver also resides various cells, including Kupffer cells (KCs) (i.e. the residing macrophages), hepatic stellate cells, sinusoidal endothelial cells, and natural killer (NK) cells that could contribute greatly to

the initiation of liver inflammation, with the consequence of recruiting other immune cells such as dendritic cells, monocytes, macrophages, etc. During both acute and chronic liver inflammation, these immune cells are activated by damage-associated molecular patterns (DAMPs), which are endogenous molecules released from damaged cells, and pathogen-associated molecular patterns (PAMPs), which are exogenous molecules derived from microbes (76). Together, PAMPs and DAMPs could act as ligands for receptors such as toll-like receptors (TLRs), and NOD-like receptors (NLRs), leading to the activation of the pro-inflammatory pathways, often times including transcription factors such as nuclear factor-kappa B (NF- κ B), stress kinase such as c-Jun N-terminal kinases (JNKs). This then leads to increased production of pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-6, IL-18, etc. that not only perpetuates the recruitment of immune cells, but also act as signaling molecule to exacerbate the already damaged parenchyma and/or further hepatocyte apoptosis. If the inflammation remains unresolved, the chronic activation of the pro-inflammatory pathways could trigger fibrotic response as the generation of myofibroblasts replace dead hepatocytes. This irreversible damage signifies the onset of declining hepatic functions.

Kupffer cells are liver-specific macrophages that reside in the liver sinusoids and constitute approximately 20% of the liver non-parenchymal cells (77). This is because Kupffer cells rapidly recognize exogenous and endogenous immunoreactive materials including LPS, FFAs, etc., and are able to initiate interactions by releasing various mediators including chemokines, cytokines, ROS, nitric oxide, etc. (78). In effect, these inflammatory mediators could recruit other immune cells such as natural killer (NK)

cells, NK T-lymphocytes, and even additional blood-derived macrophages. Much evidence suggests that activation of Kupffer cells are critical in the pathogenesis of NAFLD (79-81). As shown by experimental and human models of NAFLD, during the pathogenesis of NAFLD, an obese phenotype, due to numerous dietary factors (such as high-fat and high-fructose diets), could highly contribute to increased bacterial translocation, thus increased LPS exposure, because of altered intestinal bacteria population, and increased epithelial permeability (82-84). This increases the chance of activation of Kupffer cell by LPS-induced signaling of TLR-4. For example, TLR-4 mutant mice are defective in LPS signaling (85), and TLR-4-deficient mice are found to be protective against liver steatosis, inflammation, and fibrosis as shown by liver histology (86). It is also worth noting that FFAs are known ligands for TLR-4 (87), indicating that activation of Kupffer cells does not solely induced by LPS, but also by a FFAs-enriched environment, as in the case during obese and/or NAFLD conditions.

AMPK: A PLEIOTROPIC KINASE THAT REGULATES METABOLISM AND INFLAMMATION

AMPK is a heterotrimeric protein complex that comprises the catalytic α -subunit, and the regulatory β - and γ -subunits. AMPK is usually described as an energy on/off switch that responds in a very sensitive manner to the cellular energy status, which is the ATP status. Specifically, AMPK highly responds to the cellular ADP/ATP and AMP/ATP ratio, both of which signifies energy stress or low energy status of the cell. During times of energy stress, the increase in AMP concentration could act as an

allosteric activator by binding to the γ -subunit of AMPK, thus making it more susceptible for phosphorylation of AMPK at site Thr 172 by its upstream kinase, liver kinase B1 (LKB1); also inhibiting dephosphorylation by protein phosphatases (88). In the absence of changes in AMP concentration, AMPK can also be alternatively phosphorylated by the Ca^{2+} / calmodulin-dependent kinase kinases (CaMKKs), especially CaMKK β , in response to a change in intracellular Ca^{2+} changes (89).

As a pleiotropic kinase, upon activation of AMPK, it signals the downstream via phosphorylation of the substrates, both metabolic enzymes and regulatory proteins, thereby acutely activating or inhibiting a metabolic pathway. For example, AMPK plays important role in regulating glucose metabolism by direct phosphorylation of TBD1C1 (Tre2/Bub2/Cdc16 domain protein1), thereby promoting GLUT4 trafficking to the plasma membrane, increasing glucose uptake in skeletal muscle (90). AMPK also plays a role in regulating lipid metabolism. Particularly, AMPK phosphorylates ACC and 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, thereby inactivating fatty acid synthesis and cholesterol synthesis respectively (91,92). Since inhibition of ACC would decrease the generation of malonyl-CoA, which could inhibit carnitine palmitoyl transferase 1 (CPT1), this effect by AMPK could release the inhibition of CPT1, thereby promoting fatty acid oxidation (93). Other than glucose and lipid metabolism, AMPK is also involved in enhancing mitochondrial biogenesis via phosphorylation of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) (94), and inhibiting protein synthesis via inhibiting the mechanistic target-of-rapamycin complex-1 (mTORC1) (95).

Due to the effect in regulating metabolism, AMPK has been increasingly shown to have an anti-inflammatory effect on immune cells. Normally, immune cells such as macrophages, neutrophils, dendritic cells and T cells mainly rely on oxidative metabolism to generate ATP in their inactivated state. However, in an activated state, these immune cells would switch to rely mainly on aerobic glycolysis. For example, TLR agonist-activated dendritic cells showed a switch to aerobic glycolysis, and this is associated with reduced AMPK activation, and is inhibited by pharmacological AMPK activation (96). Further studies on macrophages indicated that downregulation of AMPK is associated with a decrease in pro-inflammatory cytokine production, such as TNF- α and IL-6, and a switch to a M2 anti-inflammatory phenotype (97). Further mechanistic studies by examining AMPK- β 1 deficient macrophages showed that the resulted increase in ACC activity and decrease in fatty acid oxidation caused an increase in the accumulation of pro-inflammatory diacylglycerol, thereby a skewing of the macrophages to a M1 pro-inflammatory phenotype *in vivo* (98).

METFORMIN ACTION IN THE LIVER

Metformin belongs to the biguanide family of anti-diabetic drug. It is originally derived from galegine present in French lilac, which was used to treat many illnesses for centuries. The effectiveness of metformin in lowering blood glucose has been recorded since the 1900s (99). It wasn't until 1998 that the UK Prospective Diabetes study, which is a 10-year randomized controlled trial, that reinforces the effectiveness of metformin not only in controlling T2DM, but also in reducing risk of cardiovascular diseases and

all-cause mortality (100). As a result, metformin is now the first-line drug that is being used worldwide for treatment of T2DM, and is the most prescribed first line anti-diabetic drug for newly diagnosed T2DM patients due to its effectiveness in reducing hepatic gluconeogenesis and in increasing skeletal muscle glucose uptake. Although metformin has been used for more than 50 years with great efficacy, the exact molecular mechanisms of how the drug works still remain under debate.

However, the current literature mostly agrees that metformin could work through transient inhibition of the mitochondrial respiratory chain complex I, thereby reducing the ratio of AMP: ATP (101). This effect then could stimulate the allosteric activation of AMPK by AMP, thereby increasing the activation/phosphorylation of AMPK by the upstream kinase, such as LKB1. Known to be a pleiotropic kinase, AMPK not only switches off anabolic pathways, but also switches on catabolic pathways. In fact, the glucose-lowering effect of metformin is due to its ability to suppresses the expression of gluconeogenic genes via AMPK interaction with several molecules such as SIRT1, CBP, SHP that could then disrupt the activities of transcriptional activators of gluconeogenic genes (102).

AMPK, other than regulating glucose metabolism, also plays a role in regulating lipid metabolism (103). For example, AMPK is able to directly phosphorylate ACC, and also is able to decrease liver X receptor- α (LXR α)-regulated expression of SREBP-1c, thus decreasing lipogenesis (104). This role of AMPK has also rendered the beneficial effect of metformin in decreasing hepatic steatosis. For example, treatment of ob/ob mice with metformin showed a marked decreased in liver size and hepatic steatosis level

(105). Also, in both rat hepatocytes (106) and human HepG2 cultures (107), metformin treatment leads to a decrease in hepatic ACC activation dependent on hepatic AMPK activation, as well as an increase in hepatic fatty acid oxidation (FAO). Furthermore, concurrent treatment with metformin and an AMPK inhibitor brings about an increase in ACC activity, along with attenuated metformin actions on suppressing hepatic lipogenesis and on increasing FAO (106). Consistently, metformin treatment significantly decreases the hepatic mRNA expressions for SREBP-1c and FAS, and concurrently increases AMPK activation (106).

Other than anti-steatosis effect, there is recent evidence that metformin could suppress liver inflammation (6), which is another important component in NAFLD. Although metformin was shown to decrease histological liver inflammation in mice induced with methionine-choline-deficient diet, the exact cellular mechanism is not very well-explained. Indeed, there is a lack of studies in the current literature that explains the cellular mechanisms of how metformin suppresses liver inflammation. Therefore, other than confirming the anti-hepatic steatosis effect of metformin, it is of particular interest to study how metformin affects the adipose tissue to indirectly improve liver steatosis and inflammation. In addition, the effects of how metformin affects macrophage status in mediating the inflammatory status of the liver will also be examined.

SUMMARY

NAFLD, a spectrum of liver metabolic diseases, is highly associated with obesity and insulin resistance, and is becoming the most common liver disease in the Western society. During the pathological state, several metabolic pathways including *de novo* lipogenesis, and FFA availability from the adipose tissue could be altered, thereby resulting in an increased liver fat accumulation. Chronic fat accumulation in the liver and pro-inflammatory mediators derived from adipose tissue could well contribute to the inflammatory progression of NAFLD, therefore, causing various complications such as NASH, fibrosis, and sometimes, carcinoma. Since there hasn't been an effective pharmaceutical managing strategy, various efforts have been undergone to study the effects of different compounds on NAFLD. With regard to this, metformin is of particular interest in our lab because of its ability to target AMPK. Because of this, metformin is not only able to improve glucose and fat metabolism, but also potentially improve inflammatory status in various tissues such as the liver and adipose tissue. In the context of NAFLD, macrophages play important role in generating cross-talk to the parenchyma in a paracrine manner, thereby affecting the inflammatory status of the liver. Therefore, it is of particular interest to study the effects of metformin in mediating various cell populations such as the hepatocytes, macrophages, and adipose tissue in improving hepatic steatosis and liver inflammation.

CHAPTER III

METFORMIN AMELIORATES HEPATIC STEATOSIS AND LIVER

INFLAMMATION IN VIVO*

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is defined by fat deposition in hepatocytes (hepatic steatosis). In generally accepted concepts, NAFLD is comprised of simple steatosis, which may be benign, and non-alcoholic steatohepatitis (NASH), which is the advanced form of NAFLD. Simple steatosis progresses to NASH when the liver develops overt inflammation and necrotic damage that are not associated with alcohol consumption. It is now recognized that NASH is a leading causal factor of cirrhosis and hepatocellular carcinoma (19,108). Additionally, hepatic steatosis is a major contributor of dyslipidemia that works with or without insulin resistance to significantly increase the incidence of atherogenic cardiovascular diseases (109). Given this, a better understanding of how to reduce hepatic steatosis and how to decrease liver inflammation are of critical importance in effectively managing NAFLD and fatty liver-associated metabolic and inflammatory diseases.

Because NAFLD is highly prevalent in obese populations (110), obesity-associated insulin resistance is considered as a factor that critically contributes to the

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development of NAFLD. Mechanistically, insulin resistance at both hepatic and systemic levels, along with hyperinsulinemia, acts to increase the expression of genes for lipogenic enzymes such as acetyl-CoA carboxylase 1 (ACC1) and fatty acid synthase (FAS) (20,111) and to decrease the expression of genes for fatty acid oxidation including carnitine palmitoyltransferase 1a (CPT1a) (112). These changes, in turn, bring about hepatic steatosis, which is considered as the primary “hit”. As indicated by the results from cultured hepatocytes, chronic fat deposition could trigger the inflammatory responses, usually considered as the “second hit”, by excessive oxidation and peroxidation. As shown by Nakamura et al., palmitate accumulation in the hepatocytes could induce pro-inflammatory response through activation of JNK by increased reactive oxygen species (ROS) production (113). Activated JNK could phosphorylate c-Jun, which then forms the activator protein-1 (AP-1) transcription factor that could increase the expression of pro-inflammatory cytokines such as TNF- α , Il-6, etc. (61). Indeed, the JNK pathway has been implicated to contribute to the development of NASH (114). Other than triggering the JNK pathway, ROS is also known to be able to stimulate the NF- κ B (115), which is another transcription factor that increases production of pro-inflammatory cytokines. As has been implicated, NF- κ B activation in the liver not only increases inflammatory status of the liver but also is responsible for the inflammatory progression of liver diseases such as liver injury, fibrosis, and hepatocellular carcinoma (116).

Metformin is a widely used anti-diabetic medicine that effectively lowers plasma glucose levels primarily by decreasing hepatic glucose production (HGP) and by

improving lipid metabolism in both liver and muscle tissues (117-120). At the cellular level, metformin activates AMPK, which serves as a key mechanism by which metformin treatment brings about a wide range of metabolic benefits (121). Though, recent evidence also suggests that metformin is capable of inhibiting hepatic gluconeogenesis, a key flux whose increase contributes to elevation of HGP and hyperglycemia, through pathway(s) other than AMPK (122,123). Although the mechanisms underlying metformin actions are more complicated than what were thought before, there is accumulating evidence that demonstrates the beneficial effects of metformin treatment on improving hepatic steatosis and on inhibiting liver inflammation (7,124). However, the exact mechanisms, particularly the anti-inflammatory effect of metformin on the liver, is unclear. Accordingly, it was hypothesized that metformin not only protects against NAFLD through direct effects on liver metabolic response, but also through decreasing liver inflammatory responses associated with decreased in JNK and NF- κ B signaling.

MATERIALS AND METHODS

Animal experiments

C57BL/6J mice were obtained from the Jackson Laboratory and maintained on a 12:12-h light-dark cycle (lights on at 06:00). At 5 – 6 weeks of age, male mice were fed a high-fat diet (HFD, 60% fat calories, 20% protein calories, and 20% carbohydrate calories) for 12 weeks and treated with metformin (150 mg/kg body weight/d, solutions in PBS) or PBS via oral gavages for the last 4 weeks. As additional controls, gender- and

age-matched C57BL/6J mice were fed a low-fat diet (LFD, 10% fat calories, 20% protein calories, and 70% carbohydrate calories) for 12 weeks and treated with PBS for the last 4 weeks. Both diets are products of Research Diets, Inc (New Brunswick, NJ) and contain the same amount of casein, L-cystein, cellulose, sucrose, soybean oil, and minerals. However, the HFD contains much more lard but none corn starch compared with the LFD. During the 12-week feeding/treatment period, body weight and food intake of the mice were recorded weekly. After the feeding/treatment regimen, mice were fasted for 4 hr before sacrifice for collection of blood and tissue samples (36,125,126). Epididymal, mesenteric, and perinephric fat depots were dissected and weighed as visceral fat content (36). Liver weight was also recorded. After weighing, part of epididymal fat was subjected to isolation of stromal vascular cells as described below. Additional adipose and liver tissue samples were either fixed and embedded for histological analyses (H&E staining) or frozen in liquid nitrogen and stored at – 80 °C for further analyses (36,127). Some mice were fasted similarly and used for insulin and glucose tolerance tests as described below. All animals received human care and all study protocols were approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Insulin and glucose tolerance tests

Mice were fasted for 4 hr and received an intraperitoneal injection of insulin (1 U/kg body weight) or D-glucose (2 g/kg body weight). For insulin tolerance tests, blood samples (5 μ l) 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 (128,129). The levels of plasma glucose were measured using an enzymatic assay kit (Sigma, St. Louis, MO).

Oil-Red-O staining and immunohistochemical analyses

Frozen liver sections were stained with Oil-Red-O as previously described (130). The paraffin-embedded liver and adipose tissue blocks were cut into sections of 5 μ m thickness and stained for the expression of F4/80 with rabbit anti-F4/80 (1:100) (AbD Serotec, Raleigh, NC) (128).

Western blots

Lysates were prepared from frozen tissue samples and cultured cells using the lysis buffer containing 50 mm HEPES (pH 7.4), 1% Triton X-100, 50 mm sodium pyrophosphate, 0.1 m sodium fluoride, 10 mm EDTA, 10 mm sodium orthovanadate, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 2 mm benzamidine, and 2 mm phenylmethylsulfonyl fluoride. After protein electrophoresis and transfer, immunoblots were performed using rabbit anti-serum as primary antibody at a 1:1,000 dilution. The

blot was followed by a 1:10,000 dilution of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody kit (Immobilon™ Western; EMD Millipore, Billerica, MA, USA) as previously described (36). GAPDH was used as a loading control. The maximum intensity of each band was quantified using ImageJ software. Ratios of P-AMPK/AMPK, P-ACC/ACC, Pp46/p46, and Pp65/p65 were normalized to GAPDH and adjusted relative to the average of PBS-treated control, which was arbitrarily set as 1 (AU).

RNA isolation, reverse transcription, and real-time PCR

The total RNA was isolated from frozen tissue samples and cultured/isolated 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) (54,126,131). The mRNA levels were analyzed for **ACC1, FAS, CPT1a, SREPB1c, IL-1 β , IL-6, TNF α** in tissue samples. A total of 0.1 μ g RNA was used for the determination. Results were normalized to 18s ribosomal RNA as plotted as relative expression to the average of PBS-treated control, which was set as 1.

Statistical methods

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

RESULTS

Metformin treatment ameliorates HFD-induced systemic insulin resistance and glucose intolerance

C57BL/6J mice were fed a high-fat diet (HFD) for 12 weeks to induce obesity (diet-induced obesity, DIO). Compared with age- and gender-matched mice that were fed a low-fat diet (LFD), HFD-fed and phosphate-buffered saline (PBS)-treated mice exhibited a marked increase in body weight (Figure 1 (A)), as well as overt hepatic steatosis and increased liver inflammatory responses (see below, Figures 2 and 3). These results demonstrate the establishment of NAFLD in obese mice. In addition, HFD-fed and PBS-treated mice displayed a significant increase in the severity of insulin resistance and glucose intolerance (Figure 1, (C) and (D)) compared with LFD-fed and PBS-treated mice. Upon treatment with metformin, body weight of HFD-fed mice remained the same compared with HFD-fed and PBS-treated mice. Also, metformin treatment did not alter food intake of HFD-fed mice (Figure 1 (B), HFD-Met vs. HFD-PBS). However, metformin-treated mice showed a significant decrease in the severity of HFD-induced insulin resistance and glucose intolerance (Figure 1, (C) and (D)), which were indicated by changes in plasma levels of glucose in response to a peritoneal injection of insulin and glucose, respectively. Thus, treatment with metformin ameliorates HFD-induced systemic insulin resistance and glucose intolerance without altering body weight in DIO mice.

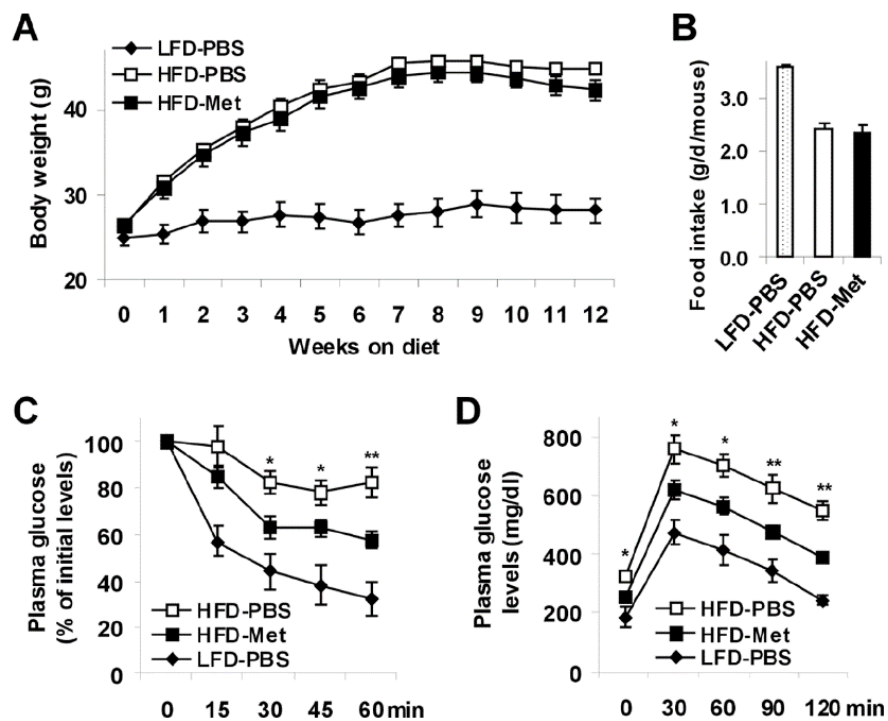


Figure 1. Metformin treatment ameliorates HFD-induced insulin resistance and glucose intolerance. Male C57BL/6J mice, at 5–6 weeks of age, were fed a high-fat diet (HFD) and treated with metformin (Met, 150 mg/kg body weight/d, in phosphate-buffered saline (PBS)) or PBS for the last 4 weeks of HFD feeding. As an additional control, gender- and age-matched mice were fed a low-fat diet (LFD) for 12 weeks and treated only with PBS for the last 4 weeks. Data are means \pm SE, $n = 6-10$. (A) Body weight was monitored weekly during the feeding/treatment regimen. (B) Food intake was calculated based on food consumption per day per mouse. (C) Insulin tolerance tests (ITT). (D) Glucose tolerance tests (GTT). For C and D, mice were fasted for 4 hr and received an intraperitoneal injection of insulin (1 U/kg body weight) (C) or glucose (2 g/kg body weight) (D). *, $P < 0.05$ and **, $P < 0.01$ HFD-Met vs. HFD-PBS for the same time point (C and D).

Metformin treatment decreases HFD-induced hepatic steatosis and increases liver AMPK phosphorylation

NAFLD is commonly associated with obesity. Consistent with increased body weight, liver weight of HFD-fed and PBS-treated mice was much greater than that of LFD-fed mice (Figure 2 (A)). Additionally, HFD-fed and PBS-treated mice displayed severe hepatic steatosis indicated by changes in liver sections stained with hematoxylin and eosin (H&E) and/or Oil-Red-O (Figure 2 (B)). Given this, HFD-fed mice, serving as a mouse model of NAFLD, were used to examine the therapeutic effects of metformin, as well as the underlying mechanisms. Compared with HFD-fed and PBS-treated mice, HFD-fed and metformin-treated mice exhibited a significant decrease in liver weight (Figure 2 (A)), which was accompanied by a marked decrease in the severity of hepatic steatosis (Figure 2 (B)). Thus, treatment with metformin effectively ameliorates hepatic steatosis in obese mice. We next examined changes in liver AMPK phosphorylation, which may underlie the beneficial effects of metformin. Compared with LFD-fed and PBS-treated mice, HFD-fed and PBS-treated mice showed a significant decrease in liver AMPK phosphorylation (Figure 2 (C)). This decrease was reversed by treatment with metformin as this was supported by the finding that liver AMPK phosphorylation in HFD-fed and metformin-treated mice was much greater than that in HFD-fed and PBS-treated mice (Figure 2 (C)). Consistently, the phosphorylation of ACC, a substrate enzyme of AMPK, was decreased in HFD-fed and PBS-treated mice compared with that in LFD-fed mice. Upon treatment with metformin, liver ACC phosphorylation in HFD-fed mice was significantly increased compared with that of HFD-fed and PBS-treated

mice (Figure 2 (C)). We also examined changes in the mRNA levels of key enzymes that critically control the development of hepatic steatosis from livers of HFD-fed mice. In terms of regulating hepatic gene expression, the effects of HFD feeding have been previously studied in rodents by a number of investigators. However, HFD feeding increases liver FAS mRNA levels when compared with a chow diet (132,133) and decreases liver FAS mRNA levels when compared with LFD feeding (134,135). Additionally, HFD feeding appears to have limited effects on liver expression of ACC and SREBP1c. In the presented study, we confirmed that HFD feeding decreased liver mRNA levels of FAS compared with LFD feeding. Meanwhile, HFD feeding did not significantly alter the mRNA levels of ACC and SREBP1c. However, within all HFD-fed mice, treatment with metformin caused a significant decrease in the mRNA levels of ACC1 and FAS compared with PBS (Figure 2 (D)). These results, together with decreased phosphorylation of liver ACC in metformin-treated mice, suggest a likely decrease in hepatic lipogenesis, which is consistent with the outcome of increased hepatic AMPK phosphorylation. Compared with LFD-fed and PBS-treated mice, HFD-fed and PBS-treated mice also displayed a decrease in liver mRNA levels of CPT1a, a rate-determining enzyme that transfers long-chain fatty acids into mitochondria for oxidation. Within HFD-fed mice, treatment with metformin tended to increase liver mRNA levels of CPT1a, but the increase was not statistically significant. Collectively, these results suggest that metformin ameliorates HFD-induced hepatic steatosis, and this effect of metformin is associated with an increase in liver AMPK phosphorylation.

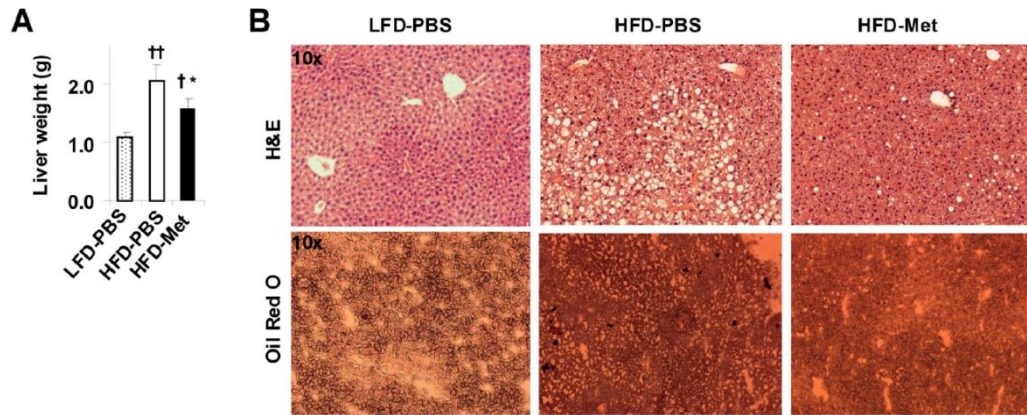


Figure 2. Metformin treatment ameliorates HFD-induced hepatic steatosis. Mice were treated as described in Figure 1. After the feeding/treatment regimen, mice were fasted for 4 hr prior to collection of tissue samples. (A) Liver weight (n = 6–10). (B) Liver histology. Top panels, H&E staining; bottom panels, Oil-Red-O staining. For bar graph A, data are means \pm SE, n = 6–8. [†], P<0.05 and ^{††}, P<0.01 HFD-PBS or HFD-Met vs. LFD-PBS (A); *, P<0.05 and **, P<0.01 HFD-Met vs. HFD-PBS (A).

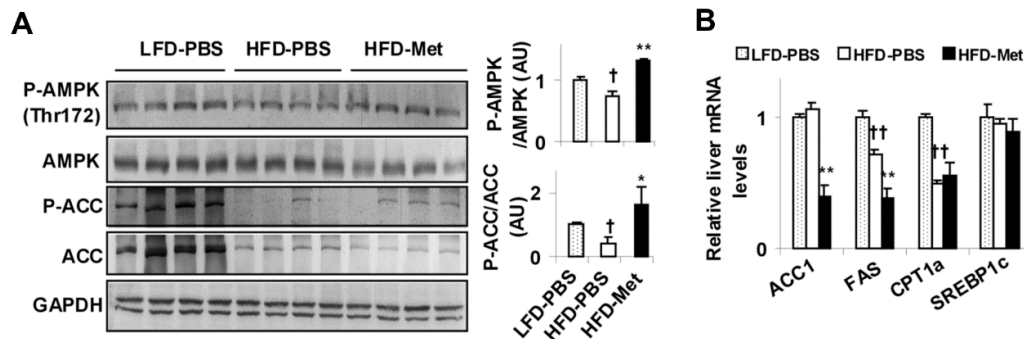


Figure 3. Metformin treatment increases liver AMPK phosphorylation in HFD-fed mice. (A) Liver AMPK signaling. Liver extracts were subjected to Western blot analyses. Ratios of phosphorylated AMPK to total AMPK (P-AMPK/AMPK) and phosphorylated ACC to total ACC (P-ACC/ACC) were quantified using densitometry and normalized to GAPDH (AU, arbitrary unit). (B) Liver mRNA levels of key lipid metabolic enzymes (genes) were analyzed using real-time PCR. For bar graph A, data are means \pm SE, $n = 6-8$. \dagger , $P < 0.05$ and $\dagger\dagger$, $P < 0.01$ HFD-PBS or HFD-Met vs. LFD-PBS (A and B); *, $P < 0.05$ and **, $P < 0.01$ HFD-Met vs. HFD-PBS (A and B).

Metformin treatment inhibits HFD-induced liver inflammatory responses

Inflammation is the key factor that drives the progression of simple steatosis to NASH. We examined the content of macrophages/Kupffer cells (F4/80⁺ cells) in livers of the mice. Unlike adipose tissue which displays a marked increase in macrophage infiltration in response to HFD feeding as established by many publications, livers of HFD-fed and PBS-treated mice contained fewer numbers of F4/80⁺ cells (Figure 4 (A), quantitative data not shown). Also, treatment with metformin tended to increase liver content of F4/80⁺ cells. These results suggest that liver content of F4/80⁺ cells is not an

ideal indicator of liver inflammatory responses. We then examined liver inflammatory signaling through JNK and NF- κ B p65 and quantified the mRNA levels of pro-inflammatory cytokines to assess liver inflammatory responses. Compared with that in livers of LFD-fed and PBS-treated mice, the phosphorylation of JNK1 (p46) in livers of HFD-fed and PBS-treated mice was significantly increased (Figure 4 (B)). A similar trend was also observed in the phosphorylation of NF- κ B p65 (Ser536) in livers of HFD-fed and PBS-treated mice compared with LFD-fed controls; although this trend did not reach statistical significance. Upon treatment with metformin, the phosphorylation of liver JNK1 (p46) was significantly decreased compared with that of HFD-fed and PBS-treated mice (Figure 3 (B)). When liver mRNA levels of proinflammatory cytokines were examined, HFD-fed and PBS-treated mice displayed a significant increase in the mRNA levels of interleukin-6 (IL-6), a proinflammatory cytokine that is abundantly expressed in hepatocytes; although HFD-fed and PBS-treated mice showed a decrease in liver mRNA levels of IL-1 β and tumor necrosis factor α (TNF α) compared with LFD-fed and PBS-treated mice. Within all HFD-fed mice, treatment with metformin significantly decreased liver mRNA levels of IL-6, as well as IL-1 β and TNF α compared with PBS (Figure 4 (C)). Together, these results suggest that metformin decreases liver inflammatory responses while improving hepatic steatosis in DIO mice.

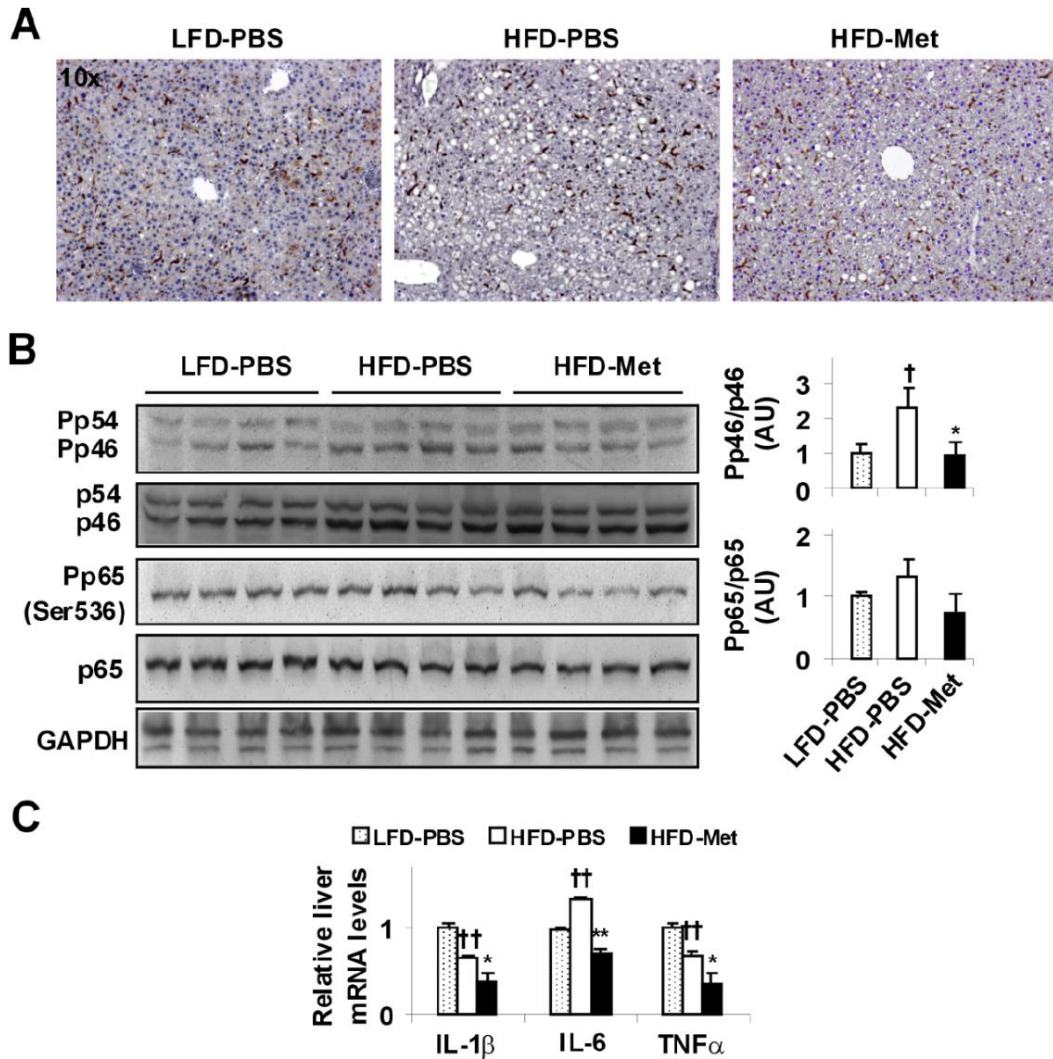


Figure 4. Metformin treatment ameliorates HFD-induced liver inflammatory responses. Mice were treated as described in Figure 1. After the feeding/treatment regimen, mice were fasted for 4 hr prior to collection of tissue samples. (A) Liver sections were stained for F4/80+ cells. (B) Liver inflammatory signaling. Liver extracts were subjected to Western blot analyses. Ratios of phosphorylated JNK1 to total JNK1 (Pp46/p46) and phosphorylated NF- κ B p65 to total p65 (Pp65/p65) were quantified using densitometry and normalized to GAPDH. (C) Liver mRNA levels of pro-inflammatory cytokines were analyzed using real-time PCR. For bar graphs (B and C), data are means \pm SE, $n = 6-8$. †, $P < 0.05$ and ††, $P < 0.01$ HFD-PBS or HFD-Met vs. LFD-PBS; *, $P < 0.05$ and **, $P < 0.01$ HFD-Met vs. HFD-PBS.

DISCUSSION

On an HFD, C57BL/6J mice developed obesity-associated insulin resistance, as well as hepatic steatosis and inflammation. Accordingly, HFD-fed mice were used as a model of NAFLD to assess the therapeutic effects of metformin. Consistent with the results obtained from both human subjects and rodent models (7,124,136), metformin treatment not only improved HFD-induced systemic insulin resistance and glucose intolerance, but also brought about a marked decrease in the severity of HFD-induced hepatic steatosis and inflammation.

Metformin is considered as an activator of AMPK. The latter, when in active form(s), exhibits an anti-lipogenic effect through suppressing hepatic expression of lipogenic enzymes including ACC1 and FAS (130). In addition, active AMPK phosphorylates and inhibits ACC1/2 (137). This leads to a decrease in the production of malonyl-CoA, which in turn releases the inhibitory effect on CPT1a to favor fatty acid oxidation. In combination, these effects of active AMPK are thought to account, to a large extent, for metformin actions on reducing hepatic steatosis. In the *in vivo* study, changes in liver AMPK phosphorylation, indicative of AMPK activity, were positively correlated with the phosphorylation of liver ACC, which indicates decreased activity of ACC, and reversely correlated with the degree of hepatic steatosis and with changes in the mRNA levels of ACC1 and FAS.

The present study also suggests a potential link between AMPK and liver inflammation that is altered by metformin. In support of this, reversal of HFD-induced decrease in AMPK phosphorylation by metformin was accompanied by decreases in the

phosphorylation of liver JNK1 (p46) and in the mRNA levels of proinflammatory cytokines. This observation was consistent with the results of a recent study in which metformin decreased liver inflammatory responses in mice fed both a methionine- and choline-deficient (MCD) diet and an HFD (124). However, the study involving MCD/HFD-fed mice did not address how metformin suppresses liver inflammation, which could originate from hepatocyte fat deposition, macrophage/Kupffer cell pro-inflammatory activation, and/or adipose tissue inflammation (54,138-140). And, these are studied and discussed further in the following chapters.

CHAPTER IV

METFORMIN DOES NOT IMPROVE ADIPOSE TISSUE METABOLIC PHENOTYPE AND INFLAMMATORY STATUS*

INTRODUCTION

Adipose tissue dysfunction is an important characteristic of obesity that can complicate the existing comorbidities associated with obesity. Adipose tissue dysfunction has also been implicated in the development of NAFLD. Indeed, this role of dysfunctional adipose tissue is highlighted by the “second hit” hypothesis. In support of this, adipocyte-specific overexpression of monocyte chemoattractant protein-1 (MCP1), an inflammatory molecule up-regulated in adipose tissue of obese mice and human subjects, mediates the effect of adipose tissue inflammation to bring about an increase in hepatic triglyceride content (141). These results and many others suggest that dysfunctional adipose tissue contributes to hepatic steatosis by increasing the delivery of fatty acid flux to the liver (19) and by impairing liver insulin signaling through adipose tissue-driven inflammation (142,143).

Metformin has been shown to be able to induce weight loss (i.e. adiposity) in both animals and humans (144-146), and this effect of metformin is found to be due to a reduction in food intake following metformin treatment. However, we do not exclude the

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possibility that metformin might act through different mechanisms other than reducing food intake. Considering that excessive adipose tissue contributes excess FFA and inflammatory mediators to the liver, causing NAFLD, we were interested in examining if metformin could act through a different mechanism, other than reducing food intake, to reduce adiposity thus, indirectly reducing the contribution of FFA and inflammatory mediators from adipose tissue to the liver during NAFLD. Therefore, using the adipose tissue collected from the DIO mice model in the previous chapter, we hypothesized that metformin treatment could decrease fat mass and the inflammatory status of adipose tissue through increasing activation of AMPK in adipose tissue.

MATERIALS AND METHODS

Animal experiments

C57BL/6J mice were obtained from the Jackson Laboratory and maintained on a 12:12-h light-dark cycle (lights on at 06:00). At 5 – 6 weeks of age, male mice were fed a high-fat diet (HFD, 60% fat calories, 20% protein calories, and 20% carbohydrate calories) for 12 weeks and treated with metformin (150 mg/kg body weight/d, solutions in PBS) or PBS via oral gavages for the last 4 weeks. As additional controls, gender- and age-matched C57BL/6J mice were fed a low-fat diet (LFD, 10% fat calories, 20% protein calories, and 70% carbohydrate calories) for 12 weeks and treated with PBS for the last 4 weeks. Both diets are products of Research Diets, Inc (New Brunswick, NJ) and contain the same amount of casein, L-cystein, cellulose, sucrose, soybean oil, and minerals. However, the HFD contains much more lard but none corn starch compared

with the LFD. During the 12-week feeding/treatment period, body weight and food intake of the mice were recorded weekly. After the feeding/treatment regimen, mice were fasted for 4 hr before sacrifice for collection of blood and tissue samples (36,125,126). Epididymal, mesenteric, and perinephric fat depots were dissected and weighed as visceral fat content (36). Liver weight was also recorded. After weighing, part of epididymal fat was subjected to isolation of stromal vascular cells as described below. Additional adipose and liver tissue samples were either fixed and embedded for histological analyses (H&E staining) or frozen in liquid nitrogen and stored at -80°C for further analyses (36,127). All animals received human care and all study protocols were approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Isolation of stromal vascular cells from adipose tissue

Adipose tissue stromal vascular cells (SVC) were isolated using the collagenase digestion method as previously described (128,147). After digestion and centrifugation, the pelleted cells were collected as SVC and subjected to FACS analyses.

Flow cytometry analysis

Adipose tissue SVC were stained with fluorescence-tagged antibodies: anti-F4/80, anti-CD11b for macrophages, and anti-CD11c and anti-CD206 for macrophage inflammatory status as previously described (148), and subjected to FACS analyses using BD FACSAria II flow cytometer (BD Biosciences, San Jose, California, USA) that

is operated by Texas A&M Health Science Center College of Medicine Cell Analysis Facility.

Western blots

Lysates were prepared from frozen tissue samples and cultured cells using the lysis buffer containing 50 mm HEPES (pH 7.4), 1% Triton X-100, 50 mm sodium pyrophosphate, 0.1 m sodium fluoride, 10 mm EDTA, 10 mm sodium orthovanadate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 2 mm benzamidine, and 2 mm phenylmethylsulfonyl fluoride. After protein electrophoresis and transfer, immunoblots were performed using rabbit anti-serum as primary antibody at a 1:1,000 dilution. The blot was followed by a 1:10,000 dilution of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody kit (Immobilon™ Western; EMD Millipore, Billerica, MA, USA) as previously described (36). GAPDH was used as a loading control. The maximum intensity of each band was quantified using ImageJ software. Ratios of P-AMPK/AMPK, Pp46/p46, and Pp65/p65 were normalized to GAPDH and adjusted relative to the average of PBS-treated control, which was arbitrarily set as 1 (AU).

RNA isolation, reverse transcription, and real-time PCR

The total RNA was isolated from frozen tissue samples and cultured/isolated 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) (54,126,131). The mRNA levels were analyzed for

IL-1 β , IL-6, TNF α , arginase 1, adiponectin, and/or resistin in tissue samples. A total of 0.1 μ g RNA was used for the determination. Results were normalized to 18s ribosomal RNA as plotted as relative expression to the average of PBS-treated control, which was set as 1.

Statistical methods

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

RESULTS

Metformin treatment does not alter HFD-induced adiposity

During obesity, dysfunctional adipose tissue critically contributes to the development of NAFLD (143). We examined changes in adipose tissue phenotype. Consistent with DIO, all HFD-fed mice displayed a marked increase in visceral fat mass compared with LFD-fed mice (Figure 5 (A)). Following treatment with metformin, HFD-fed mice did not display significant changes in visceral fat mass compared with HFD-fed and PBS-treated mice (Figure 5 (A)). Additionally, the size of adipocytes in HFD-fed and metformin-treated mice did not differ from that in HFD-fed and PBS-treated mice indicated by adipose tissue histology (Figure 5 (B)). Thus, treatment with metformin did not alter HFD-induced adiposity.

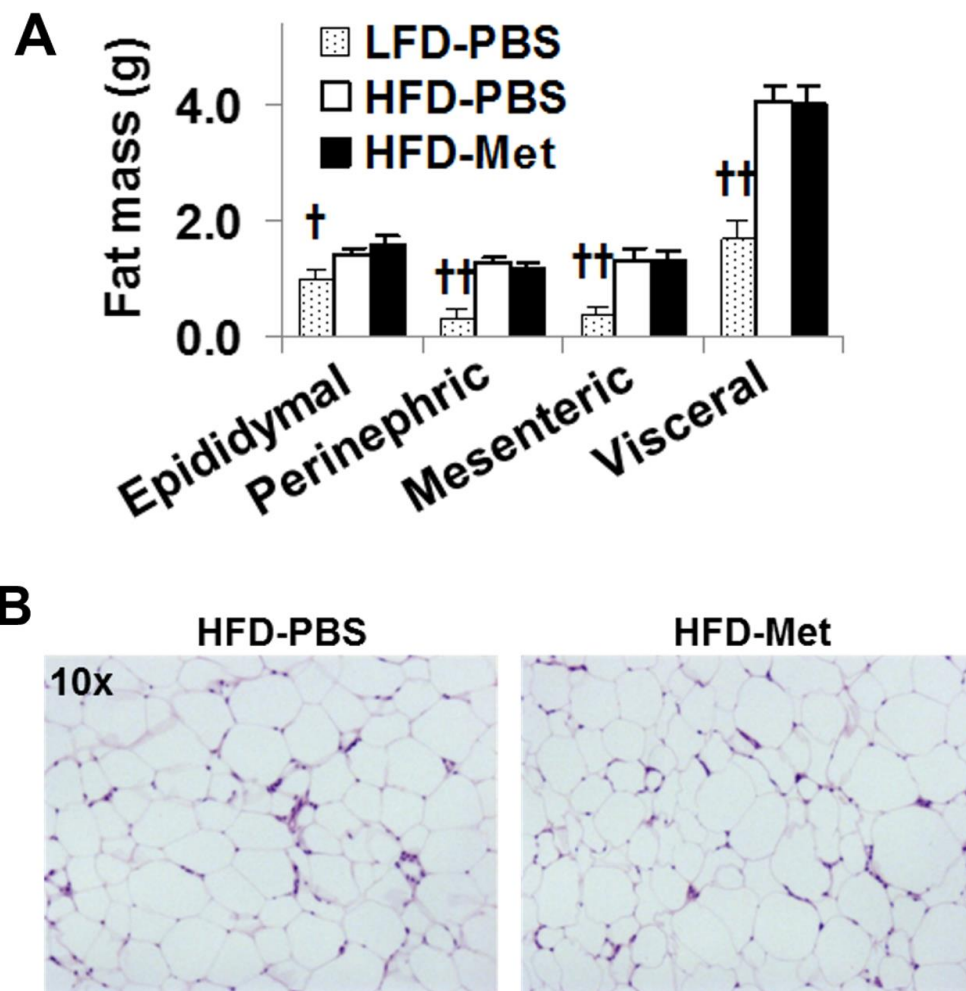


Figure 5. Metformin treatment does not alter HFD-induced adiposity. Mice were treated as described in Figure 1. After the feeding/treatment regimen, mice were fasted for 4 hr prior to collection of tissue samples. (A) Fat mass (Epididymal, perinephric, mesenteric, and visceral fat mass) (n = 6-10). (B) Adipose tissue histology (H&E staining). For bar graph (A), data are means \pm SE. [†], P < 0.05 and ^{††}, P < 0.01 HFD-PBS vs. LFD-PBS.

Metformin treatment does not alter HFD-induced adipose tissue inflammatory responses

We next examined adipose tissue inflammatory responses. As indicated by percentages of mature macrophages (F4/80⁺ CD11b⁺ cells) from the isolated adipose tissue stromal vascular cells (Figure 6 (A)), adipose tissue macrophage infiltration in HFD-fed and PBS-treated mice was markedly increased compared with that in LFD-fed and PBS-treated mice (Figure 6 (B)). However, within HFD-fed mice, treatment with metformin did not significantly alter adipose tissue macrophage infiltration (Figure 6 (B) and 6 (C)). These results were consistent with the finding that adipose tissue content of F4/80⁺ cells in HFD-fed and metformin-treated mice did not differ from that in HFD-fed and PBS-treated mice (Figure 6 (A)). Further analyses indicated that the percentages of pro-inflammatory macrophages (F4/80⁺ CD11b⁺ CD11c⁺ CD206⁻ cells) among mature adipose tissue macrophages in HFD-fed and metformin-treated mice also did not differ from those in HFD-fed and PBS-treated mice (data not shown).

Unlike changes in liver AMPK signaling, adipose tissue AMPK signaling (phosphorylation) was not altered by metformin. Additionally, metformin treatment did not alter adipose tissue inflammatory signaling, indicated by the phosphorylation of JNK1 (p46) and NF- κ B p65 (Ser536) (Figure 7 (A)). When pro-inflammatory cytokine expression was examined for HFD-fed mice, treatment with metformin did not significantly alter adipose tissue mRNA levels of IL-1 β , IL-6, and TNF α (Figure 7 (B)), as well as mRNA levels of arginase 1, adiponectin, and resistin, which all are related to adipose tissue inflammation and function. Therefore, treatment with metformin for 4

weeks appears to have limited effects on altering adipose tissue phenotype in obese mice, indicated by adiposity and inflammatory responses.

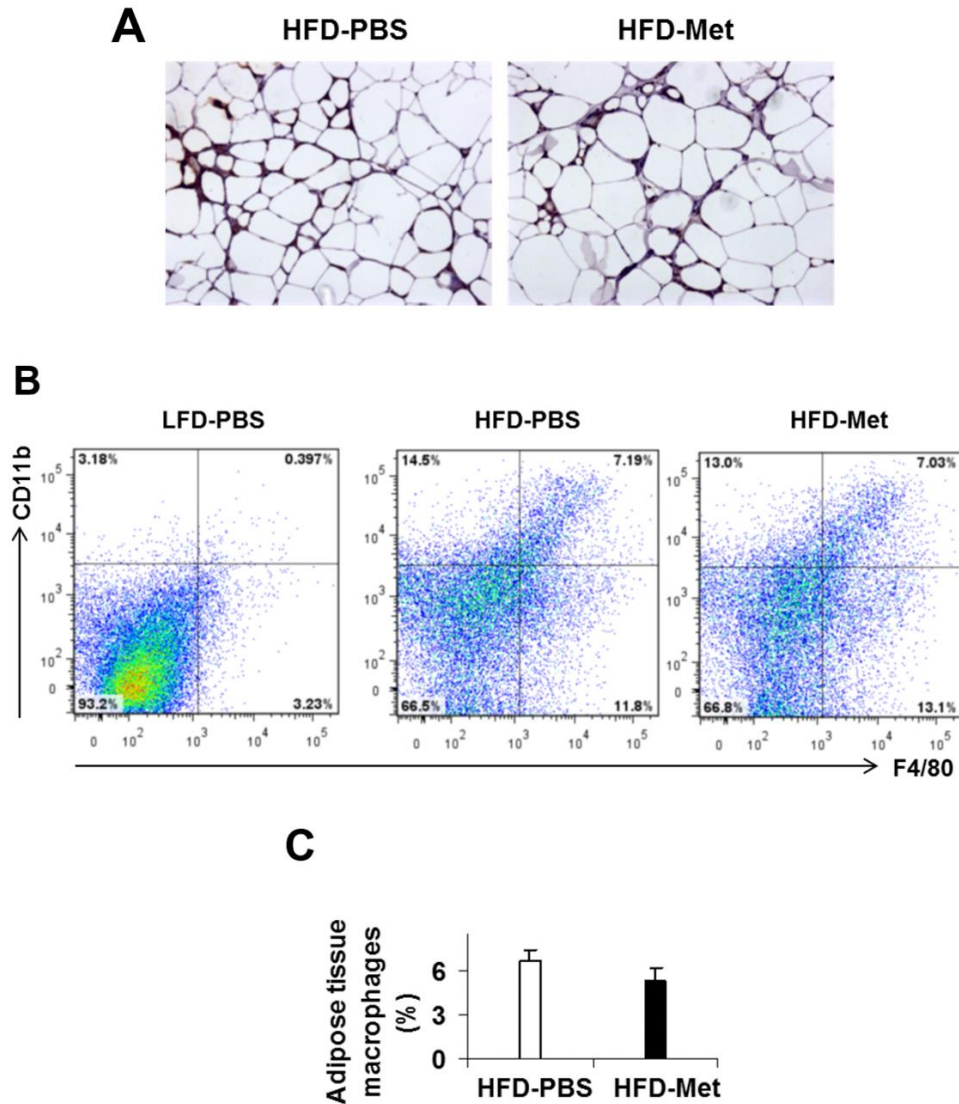


Figure 6. Metformin treatment does not alter HFD-induced adipose tissue inflammation. (A) Adipose tissue sections were stained for F4/80+ cells. (B) Adipose tissue macrophage infiltration. (C) Percentages of mature macrophages (F4/80+ CD11b+ cells) in adipose tissue stromal cells were calculated using FACS analyses (n = 4 – 6). For bar graph (C), data are means \pm SE.

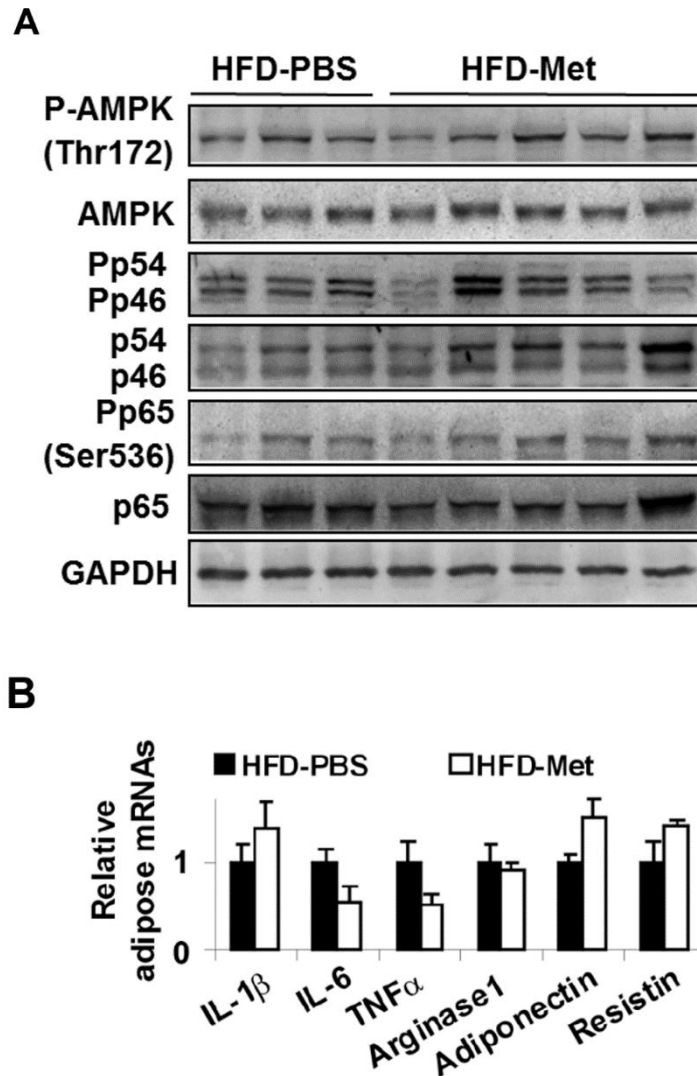


Figure 7. Metformin treatment does not alter adipose tissue AMPK status and inflammation. (A) Adipose tissue AMPK signaling and inflammatory signaling were examined using Western blot analyses (n = 4 – 6). (B) The mRNA levels of adipose genes were quantified using real-time PCR (n = 4 – 6). For bar graph (B), data are means \pm SE.

DISCUSSION

As illustrated by widely accepted concepts, dysfunctional adipose tissue during obesity contributes to the pathogenesis of NAFLD by increasing the delivery of fatty acids and inflammatory mediators to the liver to exacerbate hepatic fat deposition and inflammatory responses. Because of this, we also postulated that improved adipose tissue phenotype would contribute to the anti-NAFLD effect of metformin. However, this was not the case. In the present study, adipose tissue, unlike the liver, did not respond to metformin treatment. Notably, HFD-induced adiposity and adipose tissue inflammation in metformin-treated mice did not differ from those in PBS-treated control mice. The underlying mechanisms remained to be elucidated, but may be attributable to the effect that metformin treatment did not alter adipose tissue AMPK phosphorylation. Indeed, metformin actions on adipose tissue phenotype remain controversial. While metformin is shown to reduce body weight (adiposity) in both human and rodent models, a number of papers also demonstrate that metformin does not alter body weight, in particular in rodents fed an HFD (119,149,150). Additionally, the weight-loss effect of metformin is tied closely to a decrease in food intake, leading to a question of whether or not metformin directly acts on adipose tissue. Considering this, the beneficial effects of metformin on features of NAFLD appear to be due largely to the direct effects of metformin on the liver. What should also be mentioned is that metformin is capable of countering insulin-induced suppression of muscle fatty acid oxidation and promoting triglyceride storage in skeletal muscle (151). These effects may also contribute to the beneficial effects of metformin on features of NAFLD.

CHAPTER V

METFORMIN HAS A DIRECT EFFECT ON HEPATOCYTES AND MACROPHAGES IN IMPROVING LIPID METABOLISM AND LIVER INFLAMMATION*

INTRODUCTION

Liver inflammation plays an important role in the progression of NAFLD. Among the cell population in the liver, immune cells such as Kupffer cells that reside within the liver are highly involved in the inflammatory progression of NAFLD. Excessive activation of Kupffer cells could affect not only the non-parenchyma cells, but also the parenchyma, thereby regulating their function and phenotype (152). Therefore, depending on the source of stimuli, an altered Kupffer cell phenotype is critical in contributing towards various acute and chronic liver diseases. For example, in an obese environment, increased exposure of LPS and FFAs could induce a pro-inflammatory phenotype of macrophages, producing high levels of TNF- α that could increase hepatotoxicity and making the hepatocyte more susceptible to necrosis (153,154). Alternatively, Kupffer cells that were alternatively activated with IL-4 demonstrated improved hepatic function (155).

In the recent years, increasing studies have emphasized the role of Kupffer cells

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in the pathogenesis of NAFLD. Studies that selectively deplete Kupffer cells by administration of gadolinium chloride ($GdCl_3$), a rare metal that can inhibit mononuclear phagocytic system hence killing Kupffer cells, could protect against liver injury induced by multiple sources including alcohol (156), thioacetamide (157), ischemia (158), and most importantly, insulin resistance and hepatic steatosis (159). Likewise, depletion of Kupffer cells using chlodronate markedly blunted methionine/choline deficient (MCD) diet-induced steatohepatitis evidenced by histological improvement of the liver.

From the result of Chapter III, we demonstrated that metformin has a direct effect in suppressing liver inflammation. Therefore, in this chapter, we further hypothesized that this anti-inflammatory property of metformin in the liver not only direct targets hepatocytes, but also targets Kupffer cells in suppressing liver inflammation.

MATERIALS AND METHODS

Cell culture and treatment

H4IIE cells (rat hepatoma cells) were maintained in high glucose Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin and 100 μ g/l streptomycin as previously described (54,139). At 80% confluence, H4IIE cells were treated with metformin (500 μ M) or PBS in the presence or absence of palmitate (250 μ M) for 24 hr to induce fat deposition. To examine lipid accumulation, the treated hepatocytes were stained with Oil-Red-O for the last 1 hr. To determine changes in hepatocyte AMPK and inflammatory signaling, metformin- or

PBS-treated cells were supplemented with or without LPS (100 ng/ml) for 30 min prior to harvest. Cell lysates were prepared and used to measure the levels of AMPK, JNK1, NF- κ B p65, phospho-AMPK, phospho-JNK1, and phospho-p65 (Ser536) using Western blot analyses. To analyze hepatocyte gene expression, the total RNA was prepared from metformin- or PBS-treated cells and subjected to reverse transcription and real-time PCR.

Additional to hepatocytes, macrophages were also used to address a direct anti-inflammatory effect of metformin. Briefly, bone marrow cells were isolated from the tibias and femurs of chow diet-fed C57BL/6J mice as previously described (160). After differentiation with Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal bovine serum and 15% L929 culture supernatant for 8 days, bone marrow-derived macrophages (BMDM) were treated with metformin (500 μ M) or PBS for 24 hr in the presence or absence of LPS (100 ng/ml) for the last 30 min. Cell lysates were prepared and used to examine the inflammatory signaling using Western blot analyses. Some cells were treated with or without LPS at the same dose for 6 hr prior to harvest of RNA samples.

Oil-Red-O staining

Frozen liver sections were stained with Oil-Red-O as previously described (130).

Western blots

Lysates were prepared from frozen tissue samples and cultured cells using the lysis buffer containing 50 mM HEPES (pH 7.4), 1% Triton X-100, 50 mM sodium pyrophosphate, 0.1 M sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 2 mM benzamide, and 2 mM phenylmethylsulfonyl fluoride. After protein electrophoresis and transfer, immunoblots were performed using rabbit anti-serum as primary antibody at a 1:1,000 dilution. The blot was followed by a 1:10,000 dilution of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody kit (Immobilon™ Western; EMD Millipore, Billerica, MA, USA) as previously described (36). GAPDH was used as a loading control. The maximum intensity of each band was quantified using ImageJ software. Ratios of P-AMPK/AMPK, P-ACC/ACC, Pp46/p46, and Pp65/p65 were normalized to GAPDH and adjusted relative to the average of PBS-treated control, which was arbitrarily set as 1 (AU).

RNA isolation, reverse transcription, and real-time PCR

The total RNA was isolated from cultured/isolated 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) (54,126,131). The mRNA levels were analyzed for **ACC1**, **FAS**, **CPT1a**, **SREPB1c**, **IL-1β**, **IL-6**, **TNFα**, in cell samples. A total of 0.1 µg RNA was used for the

determination. Results were normalized to 18s ribosomal RNA as plotted as relative expression to the average of PBS-treated control, which was set as 1.

RESULTS

Metformin treatment inhibits hepatocyte fat deposition and lipogenic events

As indicated by the results listed above (Figures 2 – 4), the liver is the primary target through which metformin ameliorates NAFLD in obese mice. We next examined the direct effects of metformin on metabolic and inflammatory responses in H4IIE cells, a rat hepatoma cell line commonly used as a cell model of NAFLD (54,140). As shown in the Oil Red O staining (Figure 8 (A)), treatment of H4IIE cells with palmitate induced a significant increase in fat deposition in the absence of metformin. This effect of palmitate, however, was partially blunted by metformin treatment, indicating a direct effect of metformin on inhibiting hepatocyte fat deposition. Consistent with changes in hepatocyte fat deposition, the mRNA levels of ACC1 and FAS in palmitate-treated H4IIE cells were significantly increased compared with those in control cells (Figure 8 (B)). Following treatment with metformin, the stimulatory effect of palmitate on the expression of ACC1 and FAS was blunted (Figure 8 (B)). However, CPT1a mRNA levels were not significantly altered by palmitate in the absence or presence of metformin in H4IIE cells (Figure 8 (B)).

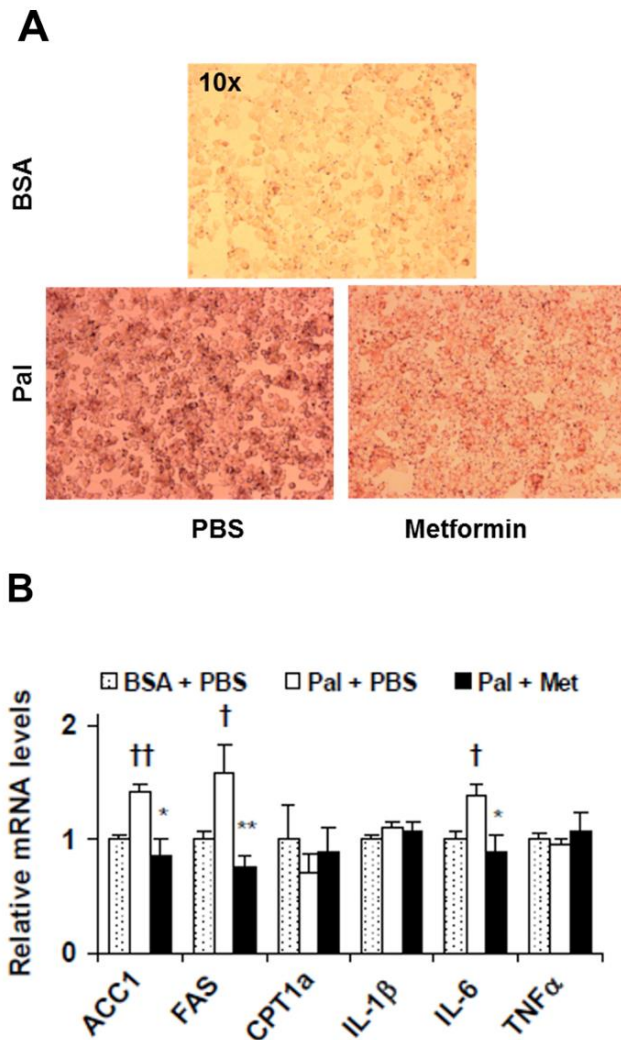


Figure 8. Metformin treatment blunts hepatocyte fat deposition. (A) Hepatocyte fat deposition. Bottom panels, cells were incubated with palmitate (Pal). (B) Hepatocyte mRNA levels were quantified using real-time PCR. For A and B, H4IIE cells were treated with metformin (500 μ M) or PBS in the presence of palmitate (250 μ M) or bovine serum albumin (BSA) for 24 hr (B) and stained with Oil-Red-O for the last 1 hr (A). For bar graphs (B), data are means \pm SE, n = 6 – 8. †, P < 0.05 and ††, P < 0.01 Pal + PBS vs. BSA + PBS (B); *, P < 0.05 and **, P < 0.01 Pal + Met vs. Pal + PBS (B).

Metformin treatment inhibits hepatocyte inflammatory signaling while increases AMPK activation

We examined AMPK signaling and inflammatory signaling in H4IIE cells. Compared with that in cells treated with control (in the absence of metformin and lipopolysaccharide (LPS)), the phosphorylation of AMPK was increased in metformin-treated cells (in the absence of LPS) (Figure 9). In the presence of LPS alone, AMPK phosphorylation was decreased compared with that in control cells (in the absence of metformin and LPS), and this decrease was partially reversed by treatment with metformin. When inflammatory signaling was examined, metformin treatment did not significantly alter the phosphorylation of JNK1 (p46) and NF- κ B p65 under the basal conditions (in the absence of LPS). However, metformin treatment significantly blunted the effect of LPS on increasing hepatocyte phosphorylation of JNK1 (p46) and NF- κ B p65 compared with that in control (PBS)-treated hepatocytes. We also examined the mRNA levels of proinflammatory cytokines. Compared with the control, the mRNA levels of IL-6 were increased in palmitate-treated H4IIE cells. This stimulatory effect of palmitate was completely blunted by treatment with metformin (Figure 8 (B)).

Together, these results suggest that metformin directly decreases hepatocyte fat deposition and suppresses hepatocyte inflammatory responses.

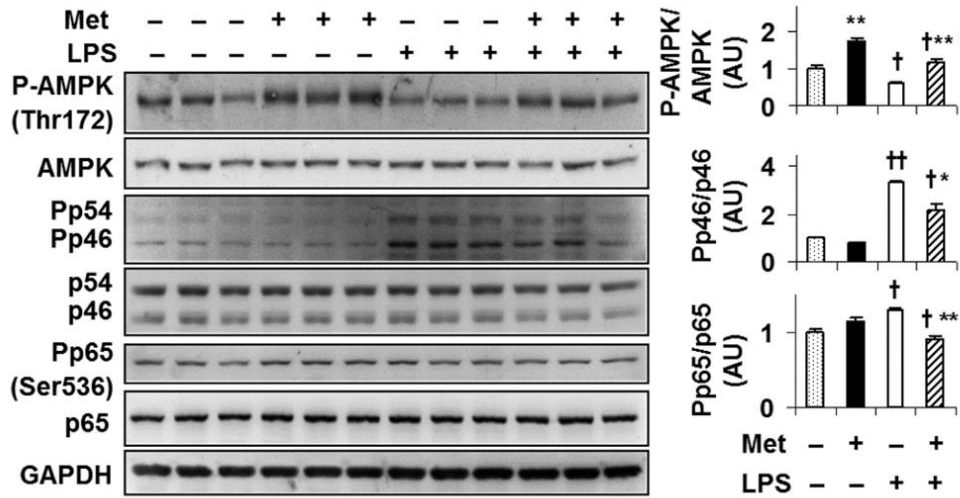


Figure 9. Metformin treatment increases AMPK phosphorylation, and decreases inflammatory responses in hepatocytes. Hepatocyte AMPK signaling and inflammatory signaling were examined using Western blot analyses. H4IIE cells were treated with metformin (500 μ M) or PBS for 24 hr in the presence or absence of LPS (100 ng/ml) for the last 30 min. Data are means \pm SE, n = 6 – 8. †, P < 0.05 and ††, P < 0.01 LPS vs. PBS (without LPS) under the same condition (with or without Met); *, P < 0.05 and **, P < 0.01 Met vs. PBS (without Met) under the same condition (with or without LPS).

Metformin treatment suppresses macrophage pro-inflammatory activation

Macrophages/Kupffer cells play a critical role in controlling the development of hepatic steatosis and inflammation (138). We examined a direct effect of metformin on macrophage pro-inflammatory activation. In the absence of metformin, bone marrow-derived macrophages (BMDM) exhibited a significant increase in the phosphorylation of both JNK1 (p46) and NF- κ B p65 (Ser536) in response to LPS stimulation (Figure 10 (A)), indicating LPS induction of macrophage pro-inflammatory activation. However, upon treatment with metformin, the effect of LPS on inducing the phosphorylation of JNK1 (p46) and NF- κ B p65 (Ser536) was partially blunted. When macrophage expression of pro-inflammatory cytokines was analyzed, the mRNA levels of IL-1 β , IL-6, and TNF α were markedly increased in control (PBS)-treated BMDM after LPS stimulation. However, in metformin-treated macrophages, the effect of LPS on increasing pro-inflammatory cytokine mRNA levels were significantly lessened (Figure 10 (B)). Together, these results suggest that metformin has a direct effect on inhibiting macrophage pro-inflammatory activation.

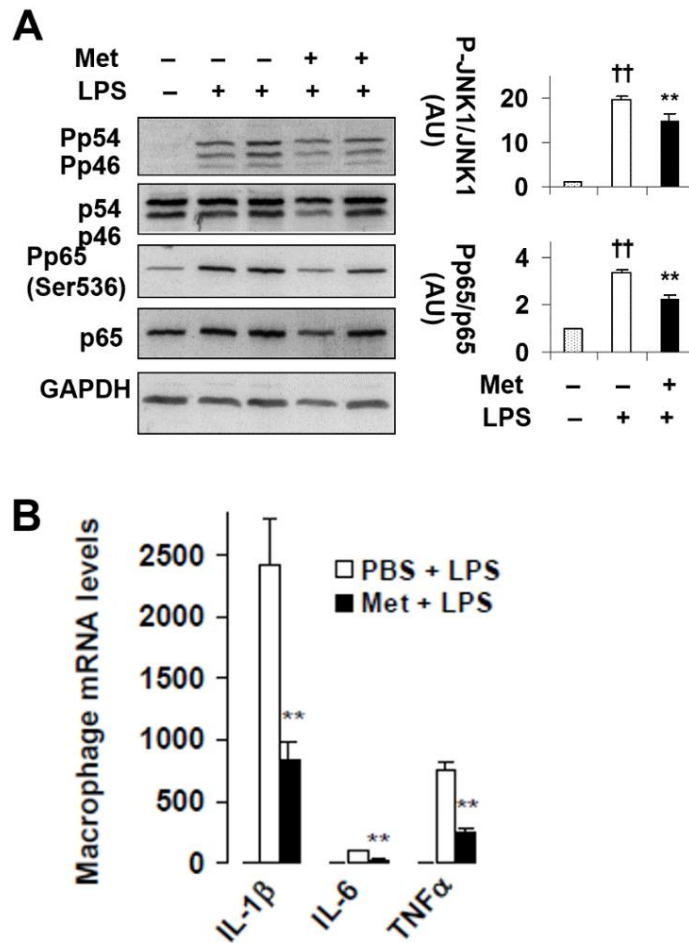


Figure 10. Metformin treatment suppresses macrophage pro-inflammatory activation. Bone marrow-derived macrophages were treated with metformin (500 μ M) or PBS for 24 hr in the presence or absence of LPS (100 ng/ml) for the last 30 min (A) or 6 hr (B). (A) Macrophage inflammatory signaling was examined using Western blot analyses. (B) Macrophage mRNA levels of proinflammatory cytokines were quantified using real-time PCR. For bar graphs (A and B), data are means \pm SE, $n = 4 - 6$. $\dagger\dagger$, $P < 0.01$ LPS vs. PBS (without LPS) in the absence of metformin (A); $**$, $P < 0.01$ Met vs. PBS (without metformin) in the presence of LPS (A) or Met + LPS vs. PBS + LPS (B).

DISCUSSION

By using an *in vitro* study, we were able to separately confirm that metformin not only could directly improve lipid metabolism and inflammation in hepatocytes, but also directly decrease macrophage inflammation. This could reflect the efficacy of metformin in decreasing hepatic steatosis and liver inflammation (by having direct effects on hepatocytes and macrophages, possibly Kupffer cells) shown in the *in vivo* study above. However, due to the fact that the macrophages in this *in vitro* study were not isolated from the liver, strictly speaking, the liver improving effect of metformin could not be claimed as improving effect in Kupffer cells in the current *in vivo* study. However, considering macrophages are of myeloid lineage, it could be assumed that metformin would have similar effects in Kupffer cells.

In addition, by comparing the *in vitro* study in this chapter and the *in vivo* study of Chapter IV, metformin treatment decreases macrophage inflammation *in vitro* but did not alter the inflammatory status of adipose tissue macrophages *in vivo*. Additional to adipose tissue inflammatory status, adiposity of HFD-fed mice was also not reduced by metformin in the present study. When adipose and liver tissues were compared, adipose tissue macrophages interacted with a highly pro-inflammatory local environment likely due to excessive fat deposition whereas liver macrophages/Kupffer cells did not. Considering this, it is also possible that a potential anti-inflammatory action of metformin on adipose tissue macrophages was offset by the pro-inflammatory effects of adipocyte-derived factors in particular saturated fatty acids (161). However, this point needs to be further validated. In addition, given that metformin did not alter adipose

tissue AMPK, it is also possible that the treatment regimen used by the present study was not sufficient enough for metformin to be effective in adipose tissue. To verify this, future studies are required to include a relative long period of time and/or a higher dose for metformin treatment. Anyhow, from the presented studies, it appears to be clear that metformin primarily targets the liver to improve features of NAFLD and this effect of metformin is independent of adipose tissue phenotype.

CHAPTER VI

METFORMIN ACTION ON IMPROVING NAFLD INVOLVES ACTIVATION OF AMPK IN HEPATOCYTES AND/OR MACROPHAGES

INTRODUCTION

AMPK is involved in glucose, lipid and protein metabolism, and is crucial in regulating the homeostasis of energy balance. It was originally discovered by its ability to inhibit fatty acid synthesis (92) and cholesterol synthesis (91) through phosphorylation of both ACC, involved in lipogenesis, and HMG-CoA reductase, involved in cholesterol synthesis, respectively. Extensive studies have then showed that AMPK plays more roles in human metabolism and diseased states than just lipid metabolism (103). Because AMPK is able to regulate hepatic lipogenesis directly through phosphorylation of SREBP-1c, thus to reduce the proteolytic cleavage and nuclear translocation of SREBP-1c, and indirectly through inhibiting SREBP-1c expression via the LXR/RXR pathway, numerous studies have confirmed that during NAFLD, AMPK is dysregulated, causing an increase in hepatic lipogenesis, thereby hepatic steatosis (162-164). One very good example is that liver AMPK activity was decreased associated with an increase in hepatic steatosis in obese, NAFLD mice induced with a HFD (165). AMPK activators such as metformin and AICAR protected against hepatic steatosis through decreasing SREBP-1c activity in experimental models (107,166).

Other than its role in lipid metabolism, AMPK has been increasingly shown to downregulate inflammation in several disease states such as acute and chronic colitis

(167), inflammation in cystic fibrosis (168), autoimmune encephalomyelitis (169), etc. More importantly, it has been demonstrated that AMPK activity is negatively associated with inflammatory markers in human adipose tissue (170). Numerous studies have then revealed that the anti-inflammatory action upon AMPK stimulation may be due to the upregulation of sirtuin 1 (SIRT1), PGC-1 α , FOXOs, and p53, thereby ultimately downregulating the NF- κ B-associated inflammatory response (171). However, limited studies have addressed the role of the anti-inflammatory effects of AMPK in the liver, though, a recent study have shown that pharmacological activation of AMPK could lead to suppression of inflammatory response in human hepatocyte cell line (172). It is also worth mentioning that a recent study has also shown that AMPK is able to regulate macrophage polarization to an anti-inflammatory phenotype under the stimulation of LPS (97).

From the result of Chapter V, we demonstrated that metformin has: (1) a direct effect on hepatocytes in decreasing hepatic steatosis by improving lipid metabolism, (2) a direct effect on hepatocytes and macrophages in decreasing liver inflammation. And, these effects were associated with an increase in AMPK activation. Therefore, in this chapter, we are interested in confirming if AMPK is necessary for the anti-inflammatory effect of metformin in decreasing liver inflammation.

MATERIALS AND METHODS

Cell culture and treatment

Primary hepatocytes were isolated as previously described (54,75). After attachment, primary hepatocytes were pretreated with AMPK inhibitor (compound C, 20 μ M) and then supplemented with metformin (500 μ M) or PBS in the presence or absence of palmitate (250 μ M) for 24 hr to induce fat deposition. To examine lipid accumulation, the treated hepatocytes were stained with Oil-Red-O for the last 1 hr. Additional primary mouse hepatocytes were pretreated with AMPK inhibitor (compound C, 20 μ M) for 30 minutes and then supplemented with metformin (500 μ M) or PBS for 24 hr to examine if AMPK is necessary for the action of metformin. Cell lysates were prepared and used to measure the levels of AMPK, ACC, phospho-AMPK, phospho-ACC using Western blot analyses.

Additional to hepatocytes, a co-culture of primary mouse hepatocytes and bone marrow-derived macrophages were used to examine the cross-talk between hepatocytes and macrophages and the involvement of AMPK. Bone marrow cells were isolated from the tibias and femurs of chow diet-fed myeloid-specific deletion of α 1AMPK mice as previously described (160), and differentiated with Iscove's modified Dulbecco's medium (IMDM) containing 10% fetal bovine serum and 15% L929 culture supernatant for 6 days. Subsequently, isolated primary mouse hepatocytes from C57BL/6J mice (WT) were added to the macrophage culture in the ratio of 10:1. On day 7, the co-cultured hepatocytes and macrophages were treated with metformin (500 μ M) or PBS for 24 hr in the presence or absence of LPS (100 ng/ml) for the last 30 min. Cell lysates

were prepared and used to measure the levels of JNK, NF- κ B p65, phospho-JNK, and phospho-p65 (Ser536) using Western blot analyses.

To verify the deletion of α 1AMPK, additional bone marrow cells were isolated from tibias and femur of chow diet-fed myeloid-specific deletion of α 1AMPK mice, and differentiated for 6 days. On day 7, macrophages were harvested and used to measure the level of AMPK.

Western blots

Lysates were prepared from frozen tissue samples and cultured cells using the lysis buffer containing 50 mm HEPES (pH 7.4), 1% Triton X-100, 50 mm sodium pyrophosphate, 0.1 m sodium fluoride, 10 mm EDTA, 10 mm sodium orthovanadate, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 2 mm benzamidine, and 2 mm phenylmethylsulfonyl fluoride. After protein electrophoresis and transfer, immunoblots were performed using rabbit anti-serum as primary antibody at a 1:1,000 dilution. The blot was followed by a 1:10,000 dilution of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody kit (Immobilon™ Western; EMD Millipore, Billerica, MA, USA) as previously described (36). GAPDH was used as a loading control. The maximum intensity of each band was quantified using ImageJ software. Ratios of P-AMPK/AMPK, P-ACC/ACC, Pp46/p46, and Pp65/p65 were normalized to GAPDH and adjusted relative to the average of PBS-treated control, which was arbitrarily set as 1 (AU).

RESULTS

AMPK is partly required for anti-steatosis effect of metformin

As previously indicated (Figure 2, 3), metformin treatment in HFD-fed C57BL/6J mice resulted in a decrease in hepatic steatosis and is associated with AMPK activation. In addition, *in vitro* study (Figure 8, 9) also confirmed the direct effect of metformin on hepatocytes in decreasing fat accumulation associated with AMPK activation. We next examined if AMPK is necessary for the anti-steatosis effect of metformin in primary mouse hepatocytes. In the absence of metformin, treatment of primary mouse hepatocytes with palmitate induced a significant increase in fat deposition (Figure 11 (A)). Similar to the result obtained in H4IIE cells (Figure 8), metformin treatment partially blunted the effect of palmitate-induced lipid accumulation. However, pretreatment of compound C (CC) abolished the anti-steatosis effect of metformin as shown by the Oil Red O staining (Figure 11 (A)).

To confirm if CC effectively inhibited the activation of AMPK, we examined AMPK signaling in primary mouse hepatocytes. Compared with that in cells treated with control (in the absence of metformin and CC), the phosphorylation of AMPK was increased in metformin-treated cells (in the absence of CC) (Figure 11 (B)). When cells are pretreated with CC, AMPK phosphorylation was not altered compared with that in control cells (in the absence of metformin and CC). However, upon metformin treatment (cells pretreated with CC), the AMPK phosphorylation paradoxically increased compared with that in the control cells (in the presence of metformin alone) (Figure 11 (B)). To confirm if our pretreatment of CC increases AMPK activity, we examined the

phosphorylation of ACC, a substrate of AMPK. Compared with that in cells treated with control (in the absence of metformin and CC), the phosphorylation of ACC was increased in metformin-treated cells (in the absence of CC) (Figure 11 (B)). However, when cells are pretreated with CC (in the absence of metformin), ACC phosphorylation was decreased compared with that in control cells (in the absence of metformin and CC). Similarly, pretreatment of CC (in the presence of metformin) resulted in a decreased in ACC phosphorylation compared with that in cells not treated with CC (in the presence of metformin) (Figure 11 (B)).

These results indicated that while pretreatment of CC paradoxically resulted in increased AMPK phosphorylation, this pretreatment of CC, however, resulted in a decrease in phosphorylation of ACC, thus increased activation of ACC, which could explain the increased in lipid accumulation upon pretreatment of CC as seen in the Oil Red O staining.

Macrophage AMPK is important in the anti-inflammatory action of metformin

Knowing that macrophages play important role in regulating liver inflammatory status, we examined the importance of AMPK in regulating the inflammatory response of macrophages, and how this will affect the inflammatory status of hepatocytes. We incubated bone-marrow derived macrophages (BMDMs) isolated from myeloid-specific deletion of $\alpha 1$ AMPK mice, and then co-cultured with primary mouse hepatocyte from C57BL/6J mice (WT). As shown in Figure 12, $\alpha 1$ AMPK deletion in macrophages was being verified. Therefore, the BMDMs and hepatocytes are co-cultured in a ratio of 1:10

in order to observe how metformin affect the inflammatory status of hepatocytes via regulating macrophage inflammation in the absence of AMPK. In the absence of metformin, the WT hepatocytes / WT macrophages co-culture exhibited a significant increase in the phosphorylation of JNK 1 (p46) and NF- κ B p65 (Ser536) in response to LPS stimulation (Figure 13 (A)), indicating LPS induction of pro-inflammatory activation. Upon treatment with metformin, the effect of LPS on inducing the phosphorylation of JNK1 (p46) and NF- κ B p65 (Ser536) was partially blunted. In the absence of metformin, the WT hepatocytes / α 1AMPK^{-/-} macrophages co-culture exhibited increase in the phosphorylation of JNK1 (p46) but not NF- κ B p65 (Ser536) compared to that of the WT hepatocytes / WT macrophages co-culture. This increase in JNK 1 (p46) phosphorylation was decreased by metformin treatment. However, when stimulated by LPS, the inflammatory signaling of the WT hepatocytes / α 1AMPK^{-/-} macrophages co-culture was not reversed by metformin treatment, as indicated by the phosphorylation status of JNK1 (p46) and NF- κ B p65 (Ser536) (Figure 13 (A)). This suggests that without α 1AMPK in macrophages, the inflammatory status of the hepatocytes / macrophages co-culture cannot be suppressed, thus emphasizing the importance of macrophage α 1AMPK in mediating the anti-inflammatory effect of metformin.

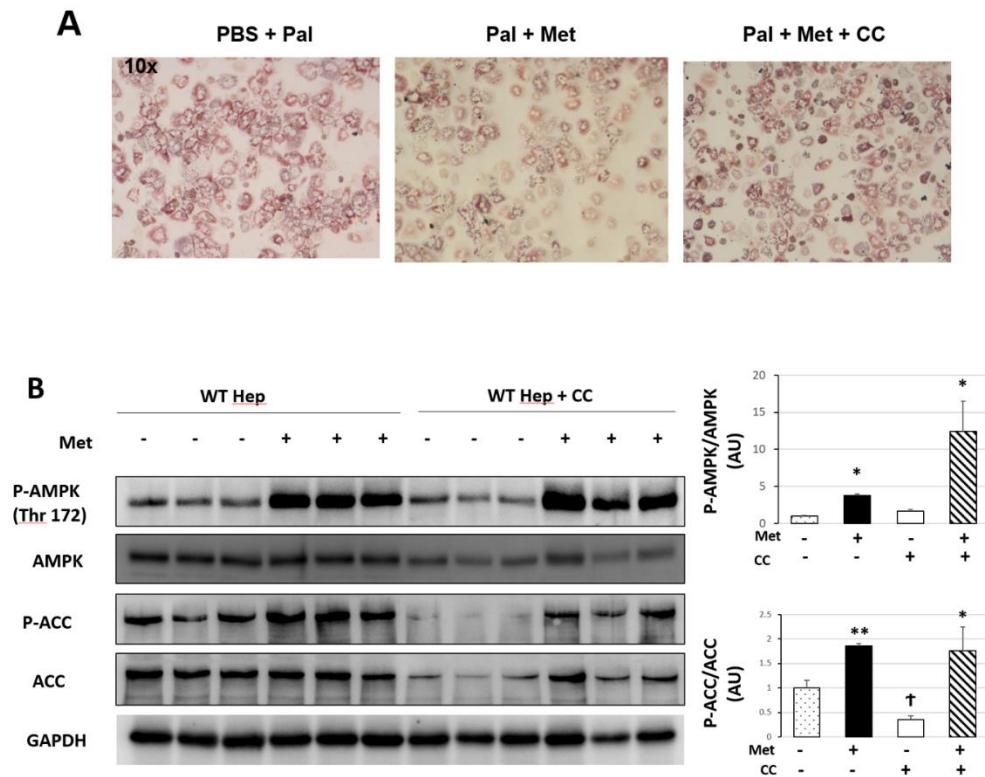


Figure 11. Treatment of CC blunts the anti-steatosis effect of metformin in primary mouse hepatocytes. (A) Hepatocyte fat deposition. Primary mouse hepatocytes were pretreated with CC for 30 min, then with metformin (500 μ M) or PBS in the presence of palmitate (250 μ M) for 24 hr and stained with Oil-Red-O staining. (B) Hepatocyte AMPK signaling were examined using Western blot analyses. Primary mouse hepatocytes were pretreated with CC for 30 min, then with metformin (500 μ M) or PBS for 24 hr. For bar graph (B), data are means \pm SE, n = 3 – 6. †, P < 0.05 without CC vs. CC in the absence of metformin (A); *, P < 0.05 and **, P < 0.01 Met vs. PBS (without metformin) in the presence or absence of CC.

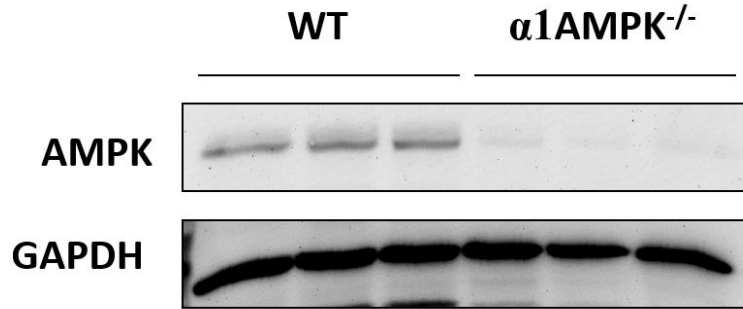


Figure 12. The confirmation of $\alpha 1$ AMPK $^{-/-}$ BMDM. AMPK expression in BMDM using Western blot analyses.

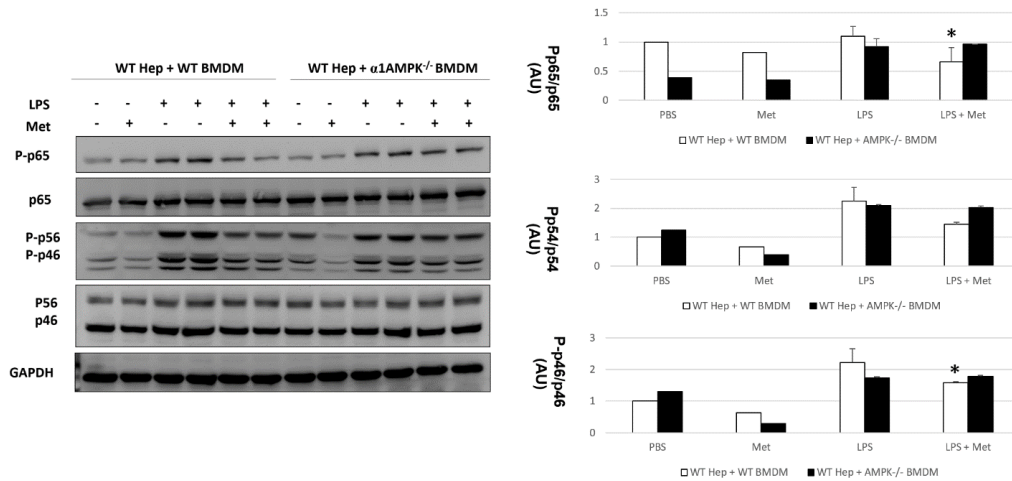


Figure 13. Metformin treatment has no effect on decreasing inflammatory signaling in WT hepatocytes / $\alpha 1$ AMPK $^{-/-}$ macrophages co-culture. WT hepatocyte / $\alpha 1$ AMPK $^{-/-}$ macrophages co-culture was treated with metformin (500 μ M) or PBS for 24 hr in the presence or absence of LPS (100 ng/ml) for the last 30 min. Inflammatory signaling was examined using Western blot analyses. For bar graphs, data are means \pm SE, $n = 2 - 4$. *, $P < 0.05$ LPS + Met vs. LPS under the condition of WT Hep + WT BMDM.

DISCUSSION

According to the literature, it is agreeable that the mechanism of action of metformin is mainly AMPK-dependent, although some studies suggested that metformin could act through AMPK-independent pathways (173). When interpreting the data from the presented study, the anti-steatosis and anti-inflammatory effects of metformin seems to be AMPK-dependent. On one hand, the inhibition of AMPK by treatment of CC blunted the effect of metformin in decreasing hepatocyte fat accumulation. On the other hand, using a co-culture system of WT hepatocytes / $\alpha 1$ AMPK^{-/-} macrophages, metformin has limited effect in decreasing the inflammatory status of the co-culture upon challenged by LPS.

When activated, AMPK could have an anti-lipogenic effect through suppressing SREBP-1c activity, thus decreasing the expression of lipogenic enzymes such as ACC and FAS. Moreover, active AMPK could phosphorylate, thus decrease the activity of ACC, one of the key enzymes of *de novo* lipogenesis. When inhibited, AMPK should decrease the phosphorylation of downstream, one of which is ACC. When phosphorylated, ACC activity decreases, leading to a decrease in lipogenesis (174). In the presented study, the treatment of AMPK inhibitor, CC, which is a cell permeable AMPK competitive reversible inhibitor widely used to study effects of AMPK signaling, resulted in an increase in fat deposition in the hepatocytes although under the presence of metformin. Interestingly, treatment of CC together with the presence of metformin resulted in even more increase in AMPK phosphorylation than that in no CC treatment. This paradoxical increase in AMPK phosphorylation remains to be further examined, but

might be due to the pretreatment of CC before treatment of metformin. Also, it is possible that the decrease in ACC phosphorylation in relative to phosphorylated AMPK upon CC treatment resulted in a decrease in fatty acid oxidation and ATP generation. The latter, as a feedback mechanism, causes an increase in AMPK phosphorylation. Consistent with this mechanism, pretreatment of CC blunted the anti-steatosis effect of metformin in the hepatocytes (Figure 11 (A)), indicating that CC possibly induces fat accumulation while bringing about an increase in AMPK phosphorylation. Considering the controversies regarding the selectivity of the inhibitory action of CC (175,176), it is also possible that CC acts through an AMPK-independent mechanism to blunt the anti-steatotic effect of metformin.

Other than altering lipid metabolism, AMPK has also been widely implicated in regulating inflammation, specifically through mediating the NF- κ B pathway (171). A recent study highlighted the importance of AMPK in regulating the inflammatory status of macrophages by showing that myeloid deletion of α 1AMPK in mice demonstrated enhanced macrophage pro-inflammation (177). Knowing that macrophage/Kupffer cells are important in regulating inflammation during NASH (178), the presented study demonstrated the importance of macrophage AMPK in mediating the inflammatory status of the environment, as well as the anti-inflammatory property of metformin, by co-culturing WT hepatocytes and α 1AMPK^{-/-} macrophages treated with metformin. In the control (WT hepatocytes / WT macrophages co-culture), metformin was able to decrease the NF- κ B signaling under LPS stimulation. However, without α 1AMPK in macrophages, metformin was unable to decrease the phosphorylation of NF- κ B p65

when challenged with LPS, likely that the pro-inflammatory status of the co-culture was not altered. Notably, the signaling of JNK 1 p46 also demonstrated a similar pattern in the metformin-treated WT hepatocytes and $\alpha 1$ AMPK^{-/-} macrophages co-culture under the stimulation of LPS. This is interesting because AMPK has also been shown to downregulate metabolic stress related-JNK activation through enhancing PGC-1 α associated mitochondria biogenesis (179,180). Together, these results provided evidence that AMPK in macrophage is important in regulating inflammation in the liver, and that AMPK is partly required for the anti-steatosis effect of metformin, whereas macrophage AMPK is important in the anti-inflammatory property of metformin.

CHAPTER VII

SUMMARY AND CONCLUSION

SUMMARY

In the presented study, C57BL/6J wild-type mice were fed a high-fat diet to mimic obesity-induced insulin resistance and hepatic steatosis as an animal model to study the beneficial effects of metformin in NAFLD *in vivo*. As expected, metformin treatment not only ameliorated insulin resistance as evidenced by an improvement in glucose tolerance test and insulin tolerance test, but also resulted in reduced hepatic steatosis as shown by liver Oil-Red-O staining, and is associated with a decrease in lipogenic event shown by an increase in phosphorylation of ACC and a decrease in mRNA expression of lipogenic enzymes such as ACC and FAS. In addition, metformin treatment also reduced liver inflammation as shown by a decrease in the phosphorylation states of NF- κ B p65 and JNK1 (p46), and a decrease in mRNA expression of pro-inflammatory cytokines such as TNF- α , IL-6, and IL-1 β . These anti-steatosis and anti-inflammatory effects of metformin were associated with increased liver AMPK activation as shown by an increase in AMPK phosphorylation.

Interestingly the hypothesis that metformin could improve adipose tissue phenotype to indirectly reduce liver steatosis and inflammation was not supported by our data as evidenced by limited changes in adiposity and adipose tissue inflammation after metformin treatment. Given this, the anti-NAFLD effects of metformin appear to be independent of improving obesity-associated adipose tissue phenotype.

Further *in vitro* investigations showed that metformin has a direct effect in (1) reducing steatosis in hepatocytes, (2) reducing inflammation in both hepatocytes and macrophages. These are confirmed by showing a reduction in fat accumulation in hepatocytes, shown by Oil-Red-O staining, a reduction in mRNA expressions of lipogenic enzymes such as ACC and FAS after metformin treatment. Moreover, a decrease in phosphorylation of NF- κ B p65 and JNK1 (p46), and a decrease in mRNA expression of pro-inflammatory cytokine IL-6 in hepatocytes suggested the anti-inflammatory effect of metformin directly in hepatocyte. This anti-inflammatory effect of metformin was associated with an increase in AMPK phosphorylation. In addition, bone-marrow derived macrophages (BMDM) provided evidence for the direct effect of metformin in decreasing inflammation in macrophages as shown by a decrease in phosphorylation of NF- κ B p65 and JNK1 (p46), and a decrease in mRNA expression of pro-inflammatory cytokines such as TNF- α , IL-6, and IL-1 β .

The present study further investigated the mechanistic insight of whether AMPK is necessary for the anti-steatosis and anti-inflammatory effects of metformin in the context of NAFLD (i.e. hepatic steatosis and liver inflammation). By using compound C (CC), an AMPK inhibitor, it was shown that the pretreatment of CC was able to attenuate the anti-steatosis effect of metformin on primary mouse hepatocytes. However, the pretreatment of CC together with metformin treatment resulted in a paradoxical increase in AMPK phosphorylation than non-CC treated hepatocytes. In addition, the involvement of AMPK in macrophages regulating the anti-inflammatory effects of metformin was further strengthened by the observation that metformin could not

decrease the inflammatory status in co-culture of WT hepatocytes / $\alpha 1$ AMPK^{-/-} macrophages challenged with LPS. As metformin was able to decrease the inflammatory status of the WT hepatocytes / WT macrophages co-culture shown by decrease in phosphorylation of NF- κ B p65 and JNK1 (p46) when challenged by LPS, metformin treatment could not produce this effect in WT hepatocytes / $\alpha 1$ AMPK^{-/-} macrophages co-culture as shown by limited changes in the phosphorylation levels of NF- κ B p65 and JNK1 (p46) of the metformin- and LPS-treated WT hepatocytes / $\alpha 1$ AMPK^{-/-} macrophages co-culture. These are of particular importance because limited studies have examined the involvement of AMPK in macrophages in regulating the anti-inflammatory effects of metformin in the context of NAFLD. By studying the involvement of AMPK in macrophage to regulate liver inflammation could not only provide a better understanding of the underlying mechanism of how metformin works, but also provide a base for further drug development that targets this aspect. For example, clearly AMPK in macrophages provided an important drug target. This can set as a platform for further discovery of bioactive compounds or a synergistic blend that could be as effective or even more effective than metformin. For instance, resveratrol, a strong antioxidant found in the skin of grapes, has been shown to activate AMPK and improve glucose metabolism, as well as have synergistic effect with metformin (181).

CONCLUSIONS

The presented study provided evidence that metformin not only is effective in improving insulin resistance, but also is effective in reducing hepatic steatosis and liver inflammation. This was supported by the results of the *in vivo* model of C57BL/6J mice model fed with high-fat diet to induce obesity-associated insulin resistance and NAFLD, and treated with metformin. Also, in the presented study, metformin did not have much effect in altering adipose tissue phenotype to indirectly improve liver steatosis and inflammation. Further *in vitro* studies confirm the beneficial effects of metformin is mainly in the liver, specifically, metformin directly decreases steatosis and inflammation in the hepatocytes, and also directly decreases inflammation in macrophages. Lastly, the involvement of AMPK in the anti-steatosis and anti-inflammatory effects of metformin was shown by AMPK inhibition attenuated the anti-steatosis effect of metformin in hepatocytes, and metformin has limited effect in decreasing the inflammatory status of WT hepatocytes / $\alpha 1$ AMPK^{-/-} macrophages co-cultures. Together, these results suggested that metformin is beneficial in ameliorating hepatic steatosis and liver inflammation, and that AMPK in macrophage is important in regulating the anti-inflammatory effects of metformin during NAFLD.

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