ADENOSINE KINASE: NUTRITIONAL REGULATION AND ROLE IN DIET-INDUCED NAFLD

A Dissertation

by

JING ZHOU

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Chair of Committee,	Chaodong Wu
Committee Members,	Joseph M. Awika
	Shaodong Guo
	Linglin Xie
Head of Department,	Stephen Talcott

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ABSTRACT

Adenosine is at the crossroad of transmethylation reactions and adenosine signaling. Therefore, by regulating adenosine concentration, adenosine kinase (ADK) has been identified as an upstream regulator of a complex metabolic network. However, ADK-based therapeutics has been largely limited to neural diseases, and the relationship between ADK dysfunction and non-alcoholic fatty liver disease (NAFLD) has never been investigated to date. Since the liver is the major site for methylation reactions, and ADK widely expresses throughout the body with the highest abundance in the liver, we hypothesize that hepatic ADK dysfunction might be a novel contributor to diet-induced NAFLD. By employing liver-specific ADK knockout and overexpression mouse models, in the present study, we first confirmed the upregulation of hepatic ADK in response to High-fat diet (HFD) feeding. We then proved a protective role of hepatic ADK knockout in aspects of HFD-induced NAFLD, including insulin resistance, excessive fat deposition, and low-grade metabolic inflammation in the liver. Additionally, we observed that hepatic ADK overexpression predisposed mice to NAFLD without an HFD challenge. Lastly, we touched on the mechanisms of action and determined that following upregulated ADK expression upon HFD feeding, the global DNA hypermethylation and subsequent silencing of genes of target, peroxisome proliferator-activated receptor α (PPARα) in specific, could be the primary mechanism underlying ADK action. Besides, the reduced adenosine signaling mediated by adenosine 2A receptor ($A_{2A}R$) took a limited effect in exacerbating the aforementioned pathological

process. Taken together, our research indicated the causal effects of hepatic ADK overexpression on diet-induced NAFLD. Even though more studies are warranted to further extend the mechanisms, ADK-oriented strategies hold huge promise in terms of effectively treating NAFLD.

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Contributors

This work was supported by a dissertation committee consisting of Professor Chaodong Wu (advisor), Associate Professor Shaodong Guo of the Department of Nutrition and Food Science (Home Department), Associate Professor Linglin Xie of the Department of Nutrition and Food Science (Home Department) and Associate Professor Joseph M. Awika of the Department of Soil and Crop Science (Outside Department).

The student independently conducted all the research for this dissertation. All the figures depicted in this dissertation will be used for future publication.

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NOMENCLATURE

ADK	Adenosine Kinase
NAFLD	Nonalcoholic Fatty Liver Disease
ATP	Adenosine Triphosphate
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
AMPK	5' Adenosine Monophosphate-activated Protein Kinase
ADA	Adenosine Deaminase
AR	Adenosine Receptor
$A_{2A}R$	Adenosine 2A receptor
SAM	S-adenosylmethionine
SAH	S-adenosylhomocysteine
SAHH	S-adenosylhomocysteine hydrolase
Hcy	Homocysteine
Met	Methionine
ENT	Equilibrative Nucleoside Transporter
DNMT	DNA Methyltransferase
cAMP	Cyclic Adenosine Monophosphate
Gi	G Protein Inhibitory
Gs	G Protein Stimulatory
WT	Wild-Type

LFD	Low Fat Diet
HFD	High Fat Diet
GTT	Glucose Tolerance Test
ITT	Insulin Tolerance Test
IR	Insulin Resistance
ACC1	Acetyl-CoA Carboxylase1
FAS	Fatty Acid Synthase
SREBP-1c	Sterol Regulatory Element-Binding Protein 1c
CPT1	Carnitine Palmitoyltransferase 1
BOH	β -Hydroxybutyric acid
IL-6	Interleukin-6
TNF-α	Tumor Necrosis Factor-α
JNK	c-Jun N-terminal Kinase
NFκB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
RT-PCR	Reverse Transcription Polymerase Chain Reaction
T2DM	Type 2 Diabetes Mellitus
CVD	Cardiovascular disease
TG	Transgenic
DKO	Double Knockout
5mC	5-methylcytosine

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CHAPTER I

INTRODUCTION

Adenosine kinase (ADK) is present in most tissues with the highest abundance in the liver [1]. ADK is the primary enzyme responsible for catalyzing the phosphorylation of adenosine to AMP: Adenosine + ATP → AMP + ADP， which is the main route for adenosine clearance [2, 3]. Therefore, ADK plays an indispensable role in maintaining adenosine homeostasis. Adenosine is a ubiquitous energy metabolite with many beneficial functions in various organ systems [4]. It is reported that adenosine, which acted as a cell-protective molecule, was released by cells mainly through ADK activity inhibition to rapidly react to harsh conditions such as hypoxia and ischemia [5-7]. ADK also plays a crucial role in the maintenance of methylation reactions [8, 9]. Adenosine is an obligatory end product of transmethylation reactions and needs to be constantly removed by ADK to maintain the transmethylation reactions [9, 10]. Due to its multiple functions, ADK dysregulation is involved in many disorders [8, 11] and not surprisingly, intensive ADK-targeted drug-developing efforts have been made to achieve anti-inflammation, pain relief as well as anti-epilepsy effects [2, 10]. However, with its highest expression in the liver, the details of ADK's involvement in the initiation/progression of NAFLD are still unknown. Recently, Xu et al. confirmed that endothelial ADK inactivation protected against endothelial inflammatory response via increasing intracellular adenosine levels and suppressing pro-inflammatory stimuli-associated hypermethylation [12]. Interestingly, ADK together with 6-phosphofructo-2-

kinase (6PFK2) belongs to the ribokinase family of proteins [2]. 6PFK2 is the enzyme responsible for the generation of fructose-2, 6-bisphosphate, which is the most powerful activator of glycolysis and simultaneously the most powerful inhibitor of gluconeogenesis [13]. Sharing similar primary and tertiary structures, ADK might also have some effects on glucose homeostasis. Notably, ADK inhibition has been proposed to treat diabetes for its potential capacity to promote islet beta-cell replication in a cell-type selective way [14]. Moreover, our preliminary data suggest that hepatic ADK protein levels significantly increased in mice fed with a high-fat diet (HFD) compared to mice fed with a low-fat diet (LFD). Though encouraging, the nutritional regulation of ADK and the subsequent events have not yet been established. Therefore, we propose to explore the function of ADK in response to different nutritional regulation, as well as its role in diet-induced NAFLD via specific knocking out or overexpressing ADK in mice liver.

CHAPTER II

LITERATURE REVIEW

Introduction

Adenosine kinase (ADK) is a ribokinase that catalyzes the phosphorylation of adenosine to AMP using ATP as a phosphate donor[10]. Another well-studied ribokinase is 6-phosphofructo-2-kinase (6PFK2), responsible for the generation of fructose-2, 6-bisphosphate, which is the most powerful activator of glycolysis and simultaneously the most powerful inhibitor of gluconeogenesis[13]. Therefore, ribokinases, together with hexokinases and galactokinases, belong to the sugar kinase family, and all play crucial roles in cellular metabolism[15].

Increasing studies have demonstrated that ADK serves important roles in diseases, via a combination of adenosine receptor-dependent and/or -independent mechanisms [5, 10, 16-18]. ADK-induced fluctuations in adenosine homeostasis may activate adenosine-associated G-protein coupled receptors (GPCRs) [19], alter energy metabolism directly [20] or through AMP-activated protein kinase (AMPK)-dependent pathways [21], and regulate DNA or histone methylation [22, 23]. The functions of ADK in neuroscience such as epilepsy [18], pain [4], traumatic brain injury [24], to name some examples, have been extensively studied. There are several thorough review articles about ADK and neural diseases available [10, 25-27], so I have not attempted a comprehensive coverage of this topic, but rather have limited my review to aspects other than neural diseases. The review in this article mainly focuses on the role

of ADK in the pathophysiological processes of cardiovascular disorders, diabetes, metabolic bone diseases, and cancer, as there is emerging evidence indicating that ADK dysfunction is widely involved in each of them.

Physiologic role of ADK

The concentration of adenosine is normally maintained at a narrow range of 30-200nM through a complex network [28]. Adenosine is generated from the dephosphorylation of extracellular ATP via CD39 and CD73 [29, 30], and from the dephosphorylation of intracellular AMP by 5'-nucleotidase [31], as well as from the conversion of intracellular S-adenosyl homocysteine (SAH) derived from the methionine cycle [9, 32]. Adenosine can be transported in and out of cells dynamically through equilibrative nucleoside transporters (ENTs) [33]. The major routes of intracellular adenosine removal are based on either deamination to form inosine via adenosine deaminase (ADA) or phosphorylation to AMP via ADK [34]. ADA deficiency is known to be involved in many immune deficiency disorders [35-37]. However, the biological functions of ADK and consequences of ADK dysfunction remain largely obscure. Since the K_m of ADK for adenosine is much lower than that of ADA [38], ADK is the principal intracellular enzyme responsible for removing adenosine and subsequently restraining the efflux of adenosine into the extracellular environment (Fig. 1). For instance, it is reported that up to 90% of adenosine in the cardiomyocyte was converted to AMP by ADK, with a small portion converted to inosine by ADA [39], thus dramatically decreasing the adenosine release from the cardiomyocyte under basal conditions.

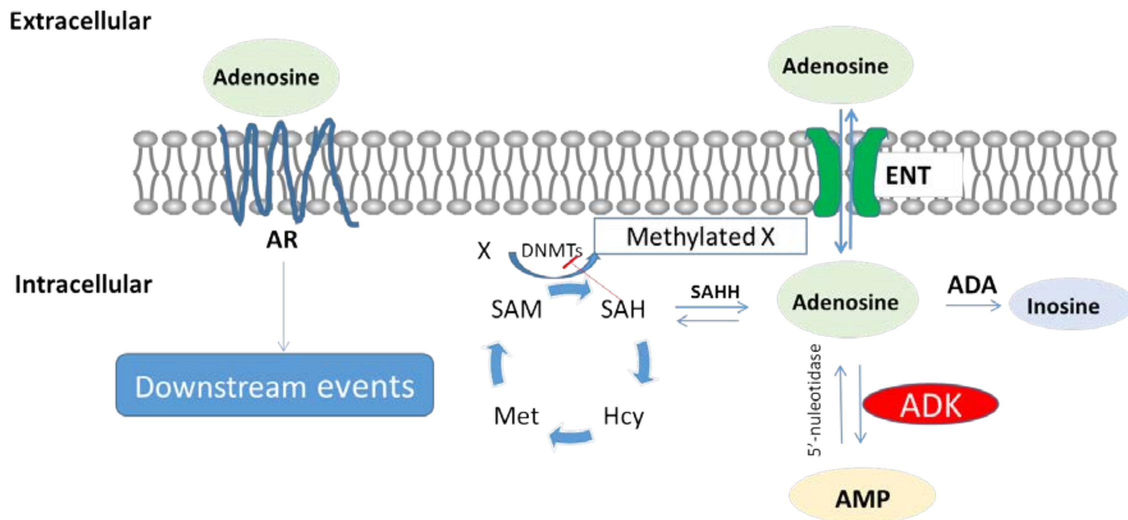

Figure 1: Physiological role of ADK.
Physiologically, adenosine can be formed through dephosphorylation of AMP. It is also an obligatory end product of transmethylation reactions. Adenosine can be transported in and out of cells through equilibrative nucleoside transporters (ENTs). Extracellular adenosine regulates downstream events by activation of four types of adenosine receptors (ARs). Intracellular adenosine should be constantly removed to maintain the transmethylation reactions. The major routes of adenosine removal are based on deamination to form inosine via adenosine deaminase (ADA) or phosphorylation to AMP via ADK. Since the K_m of ADK for adenosine is much lower than that of ADA, ADK is the principal enzyme responsible for regulating the level of adenosine. Therefore, by regulating adenosine, ADK can be identified as an upstream regulator of complex metabolic networks. SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; SAHH: S-adenosylhomocysteine hydrolase; Hcy: homocysteine; Met: methionine; X: DNA; DNMT: DNA methyltransferase.

ADK and DNA methylation

Epigenetic reprogramming is a phenomenon that impacts gene expression without changing the DNA sequence; this is due to DNA methylation, histone modifications and regulation by non-coding RNAs [40, 41]. DNA methylation is predominantly found in cytosine bases of the CpG islands [42]. Generally, DNA methylation is negatively

related to gene expression[43]. That is, hypermethylation of the CpG islands in the promoter region of a gene represses its expression and vice versa. DNA methylation requires a methyl group donated from S-adenosylmethionine (SAM), a process catalyzed by DNA methyltransferases (DNMTs) [44], and the resulting product, S-adenosylhomocysteine (SAH) is further cleaved to adenosine and homocysteine by SAH hydrolase (SAHH). The latter is a reversible reaction and SAHH favors SAH formation under physiological conditions. Critically, SAH has been proven to be a substrate inhibitor for DNMTs[45]. Therefore, the methylation capacity would be perturbed if SAH accumulates resulting from the failure of adenosine being constantly removed; the methylation capacity would be exaggerated if SAH decreases because of the downstream adenosine concentrations being too low. Since ADK is the primary enzyme responsible for the clearance of adenosine, the fluctuations of ADK activity would heavily influence the transmethylation reactions. ADK, therefore, is closely related to epigenetic reprogramming, which has been involved in human health and disease [46].

ADK and adenosine receptors

Currently, it is well established that after adenosine is released from cells or generated in extracellular space, it diffuses into the surroundings, where it binds to adenosine receptors, which are GPCRs denoted as A_1, A_{2A}, A_{2B}, and A_3 [47]. Of the four adenosine receptors, the A_1 and A_3 receptors are coupled to Gi (inhibitory) proteins, which can inhibit adenylyl cyclase and subsequently decrease intracellular levels of cyclic AMP (cAMP). In contrast, the A_{2A} and A_{2B} receptors are coupled to Gs (stimulatory) proteins and lead to opposite effects resulting in upregulated cAMP levels

[48]. Also, all adenosine receptors are coupled to mitogen-activated protein kinase (MAPK) signaling pathways, including ERK1/2, PI3K, PKA, JNK, MEK1, P38 kinase, etc. [49]. Furthermore, A_1R and A_3R can stimulate the release of calcium ions [50]. It is reasonable to speculate that unrestrained adenosine efflux due to ADK dysfunction would result in complex consequences by activating certain types of adenosine receptors on the cell membrane. Due to the abundance and redundancy of adenosine receptors throughout the body, the outcomes would be tissue/organ-specific [16, 51]. Finally, after adenosine receptor activation, adenosine moves away from the receptor and is rapidly transported into the cells predominantly through ENT-1 and ENT-2 [52, 53].

ADK and energy metabolism

ADK is the primary enzyme responsible for catalyzing the phosphorylation of adenosine to AMP: Adenosine + ATP $\rightarrow$ AMP + ADP, which is subsequently converted to ADP and ultimately to ATP. AMPK is the enzyme that monitors cellular energy status by sensing increased ratios of AMP/ATP and ADP/ATP [54]. Given the series reactions mentioned above, ADK might play a role in maintaining energy homeostasis by promoting downstream AMPK activation and increasing the adenylate pool [55]. In line with this concept, there was a 35%-40% reduction in AMP, ADP, and ATP levels in liver tissue homogenates from whole-body ADK ablation mice [1]. In addition, AMPK is a crucial energy sensor, but its function is not limited to promoting ATP production. AMPK can also regulate glucose, lipid, and protein metabolism, autophagy, and mitochondria biogenesis, as well as functions beyond metabolism [56]. The involvement of AMPK makes the function of ADK even more unpredictable and fascinating.

Moreover, ADK inhibition was reported to cause deep hypothermia in conscious mice in a receptor-independent manner, suggesting an effect of ADK in body-temperature regulation [57]. This might be a consequence of the reduction of adenylate pool.

Cell/Tissue-specific gene manipulation mouse models

Most diseases are originated in one or more specific tissues/organs. Cardiovascular disorders primarily involve the heart and vascular tissues; diabetes mainly results from β-cell mass reduction or β-cell dysfunction in pancreatic islets; osteoporosis originates from the bones; etc. It is significant to study a disease in a tissue/organ- specific condition because this makes the establishment of cause and effect easier and ensures the potential medications being explored are better targeted to the tissue in question and consequently have fewer side effects. Whole-body ADK deficiency in human and rodents has been reported to cause detrimental issues such as global development delay, encephalopathy, liver dysfunction, and even premature mortality [1, 8, 9, 11, 23]. However, cell/tissue-specific ADK disruption demonstrated no harmful, if not beneficial, effects to rodents. Since ADK is ubiquitously expressed, there is a necessity to perform studies of its functional role by specific manipulation of its gene expression in different tissues/organs.

Cre-LoxP technology

Cre-LoxP technology makes cell/tissue-specific gene knockout (KO) or overexpression possible, and it is commonly used in all sorts of biomedical research areas. This technology involves two basic mouse models. One carries a loxP-flanked

sequence of interest, and the other one expresses Cre recombinase under the control of a cell/tissue-specific promoter. When the two mouse models breed with each other, Cre-mediated recombination will result in deletion of the flanked genome, resulting in cell/tissue-specific gene KO mouse models [58]. Less commonly, when the loxP-flanked sequence is a stop code of a gene of interest, a cell/tissue-specific gene overexpression mouse model will be generated.

Depending on the targeted tissues, different transgenic recombinase-expressing mouse models could be employed. Alb-Cre mice are a mouse line in which Cre is selectively expressed in albumin expression hepatocytes; therefore it is liver-specific [59]. Adiponectin and aP2 (fatty acid binding protein 4) -Cre are adipose tissue-specific [60], LysM (lysozyme 2)-Cre is myeloid cell-specific [61], MHC (myosin heavy chain)-Cre is heart-specific [62], cdh5-Cre is vascular endothelial cell-specific [63], etc. Most of these mouse models are commercially available. Notably, there might be leaking effects depending on the specificity of the promoter. For example, RIP-Cre is a rat insulin promoter driven-Cre recombinase. It is supposed to specifically delete genes in islet beta cells, but it also has effects on hypothalamus [64]. This is contrary to MIP-Cre, which is a mouse insulin promoter with higher specificity.

Cell/Tissue-specific ADK knockout mouse models

The introduction of cell/tissue-specific ADK deletion mouse models has led to the identification of its role for different disorders. Several cell/tissue-specific ADK KO mouse models have been successfully generated. For these mouse models, ADK-floxed mice ($ADK^{f/f}$) were first generated by insertion loxP sites to both sides of ADK exon

seven. $ADK^{f/f}$ mice were then crossed with the above-mentioned Cre mice to get cell/tissue-specific mouse models. So far, an ADK KO in endothelial cells mouse model was achieved by cross-breeding Cdh5-Cre transgenic mice with $ADK^{f/f}$ mice [12]. A macrophage-specific ADK KO mouse model was generated by breeding LysM-Cre mice with $ADK^{f/f}$ mice [65]. Furthermore, pancreatic β cell-specific ADK KO was acquired by crossing $ADK^{f/f}$ mice with Ins2-Cre mice [66]. These mouse models are valuable tools for facilitating our research and understanding in a cell/tissue-specific level. Notably, not only are some cell/tissue-specific ADK KO mice well-tolerated and liable, but also they behave normal and no detectable difference to wild-type mice from the same background without metabolic insults. These mouse models are of great value for us to investigate ADK and metabolic diseases.

ADK and metabolic disease

Due to the multiple functions of ADK in metabolism, it is not surprising to see its involvement in various metabolic diseases. The role of ADK in neural diseases and the mechanisms of action have gained much attention and have been extensively exploited in the past decades [25, 67]. However, the role of ADK in other metabolic disease has long been underappreciated until recently. Among them, cardiovascular diseases, diabetes, bone disorders, and cancer are fields of great interest.

ADK and cardiovascular disorders

The importance of adenosine receptor-dependent pathways in vascular disorders has been appreciated for decades and it has been demonstrated that extracellular

adenosine binds and activates $A_{2A}R$ and/or $A_{2B}R$ on macrophages and subsequently diminishes foam cell formation [68]. Emphasis has been long placed on the necessity of adenosine receptor signaling for the vascular growth effect of adenosine. Moreover, the suppression effect of adenosine receptors, especially $A_{2A}R$, on proinflammatory cytokines production has been reported repeatedly [69]. Similarly, it has commonly been assumed that A_1R and $A_{2B}R$ activation modestly attenuate cardiomyocyte hypertrophy, while A_3R opposes these effects.

However, adenosine clearance is primarily controlled intracellularly by ADK, therefore, ADK might also critically participate in the regulation of cardiovascular diseases (CVD). This is an emerging area where Huo's research group from Augusta University takes the leading place. They conducted intensive studies within the past years with various genetic and engineered mouse models. First, their research with the endothelial cell-specific ADK KO mouse model exhibited an anti-inflammatory effect of ADK deficiency in endothelial cells through decreasing the levels of methylated histone 3 on lysine 4, which could transcriptionally activate gene expression for vascular inflammation [12]. Furthermore, they expanded their study to the epigenetic regulation effect of ADK ablation on various angiogenesis models including the development of retinal and hindbrain vasculature and skin wound healing [70]. This set of studies demonstrated that upon the hypoxia challenge *in vivo* and *in vitro*, ADK activity was inhibited. This led to the promoter region of pro-angiogenetic genes, VEGFR2 in particular, hypomethylated, and thus promoting angiogenesis [70]. Most recently, the same group proposed a novel role of macrophage ADK inhibition in the treatment of

atherosclerotic diseases. The data from their study indicated that myeloid cell-specific ADK deletion or inhibition led to adenosine accumulation in the macrophages of $ApoE^{-/-}$ (Apolipoprotein E Deficient) mice. Adenosine accumulation subsequently results in less ATP-binding cassette transporter G1 (ABCG1) gene methylation and thus more expression. ABCG1 upregulation further promoted the cholesterol efflux, followed by less foam cell formation, and ultimately prevented atherosclerotic diseases [65]. Moreover, their *in vitro* data further confirmed that in ADK-deficient macrophages the foam cell formation induced by ox-LDL was affected by the treatment of neither $A_{2A}R$ nor $A_{2B}R$ antagonist, which indicated the effect of ADK inhibition is predominant in regulating foam cell formation [65]. This effect was probably strong enough to offset the detrimental effects of adenosine receptor antagonists.

A series publication from Huo's laboratory suggests a protective role of ADK inhibition in vascular diseases. This concept is in line with the protective effects of ADK inhibition on cardiomyocyte hypertrophy from Fassett et al. They believe ADK downregulation mediated the effect of adenosine on attenuating cardiomyocyte hypertrophy, at least in part, through suppression of Raf signaling to mTOR/p70S6k, in which process, adenosine receptors played a less prominent role [39]. Another *ex vivo* study from Davila et al. suggested a beneficial role of ADK inhibition in coronary arteriole dilation in patients with heart failure with preserved ejection fraction. Their *in vivo* study with the genetic endothelial cell-specific ADK ablation mouse model and in *vitro* study with pharmacologic ADK inhibition demonstrated a $Ca2^{+}$-dependent

endothelial hyperpolarization mechanism which could be responsible for ADK deletion-induced coronary arteriole dilation [71].

Collectively, ADK modulation can influence multiple cellular participants in the formation of CVD via novel epigenetic pathways and adenosine receptor-dependent mechanisms to some extent. ADK is likely a promising target for the prevention and treatment of CVD.

ADK and diabetes mellitus (DM)

DM is a constant hyperglycemia status, which progressively leads to diffuse vascular disease and multiple organ dysfunctions. DM is primarily attributed to insufficient pancreatic β cell mass or function [72]. While pharmacological agents promoting insulin secretion from β cells have been available for diabetic patients, the prerequisite of functional β cells in the patients limits the clinical application of medications in this category [73]. Excessive overload could possibly accelerate the β cell mass reduction in the long run. Therefore, a better approach for DM is of great interest.

Interestingly, ADK, together with 6PFK2 belongs to the ribokinase family of proteins. 6PFK2 is a well-studied enzyme responsible for the generation of fructose-2, 6-bisphosphate, and thus promotes glycolysis and inhibits gluconeogenesis. Sharing similar primary and tertiary structures, ADK might also have some effects on glucose homeostasis and DM. This assumption is supported by several lines of evidence. Mechanistic studies have shown up to a 4-fold increase in ADK transcriptional activation hours after the incubation of the splenocytes isolated from diabetic rats with 10 nM insulin [74]. This insulin-induced ADK overexpression was likely to be the

mitogen-activated protein kinase (MAPK) pathway-dependent. Although Type 2 diabetes mellitus (T2DM) is accompanied by insufficient insulin secretion in the late stage, hyperinsulinemia is an early adaptation to insulin resistance. Therefore, hyperinsulinemia in early T2DM could upregulate ADK expression, which in turn contributes to the progression of T2DM.

Moreover, ADK inhibition has been proposed to treat diabetes due to its potential capacity to selectively promote primary islet beta-cell replication via mTOR activation in a cell-autonomous manner [14]. This conception is reiterated in a case report paper from Staufner et al. In the cohort they studied, most patients with ADK deficiency have recurrent and/or severe hypoglycemia likely due to hyperinsulinism [8]. However, human β cell proliferative capacity tends to diminish with age, so is the effect of ADK inhibition on β cell replication age-sensitive? Also, whether ADK inhibition can promote the replication of β cells under diabetogenic conditions as it did for the basal conditions? These questions were partially answered by Yu and Huo et al. With *in vivo* studies utilizing a pancreatic β cell-specific ADK deficiency mouse model, they demonstrated that pancreatic β cell-specific ADK ablation improved glucose metabolism, promoted β cell proliferation, and increased the β cell number in mice at the age of 4 weeks (weanings) but not at the age of 9-12 weeks (adults) [66]. Moreover, for adult mice, pancreatic β cell-specific ADK knockout protected from streptozotocin (STZ) -induced β cell damage and hyperglycemia [66].

Furthermore, Navarro G. et al. first exhibited that disruption of ADK expression

in β cells protected against diabetogenic insults with constitutive and conditional disruption mouse models, respectively. On one hand, for the constitutive ADK deficient mice, the intraperitoneal glucose tolerance test was subtle but significantly improved with aging. On the other hand, for both models, glucose tolerance, β cell function, and β cell mass were improved upon HFD challenge [75]. Surprisingly, no phenotype has ever been identified in those unchallenged mice, suggesting a role for ADK in modulating the adaptive β cell response to diabetogenic insults [75]. Indeed, identification of ADK inhibition-based strategies to promote *in vivo* β cell proliferation and increase β cell mass while retaining its insulin-secreting function is an attractive therapeutic strategy for diabetes.

Besides, approximately one out of four patients with DM develop renal injury [76], which is the primary cause of the end-stage renal disease (ESRD) worldwide [77]. Pye et al. reported a reno-protective effect of ADK inhibition, which is likely mediated by attenuating the oxidative stress and inflammation, in streptozotocin-induced diabetic mice [78]. In this regards, ADK could be a novel therapeutic target to halt the progression of diabetic nephropathy.

ADK and bone disease

A critical role for adenosine and its receptors in bone homeostasis including osteoclast/osteoblast differentiation, bone formation and resorption, and chondrocyte and cartilage homeostasis has been well documented [79]. Though still controversial, scholars tend to believe that adenosine signaling through A_1R and $A_{2B}R$ enhances

osteoclastogenesis and through $A_{2A}R$ inhibits osteoclastogenesis [80]. Moreover, the activation of A_3R suppresses osteoclastogenesis presumably due to its anti-inflammatory property [79]. Since the concentration of adenosine is tightly regulated by ADK, and the subsequent adenosine signaling would be subjected to the alteration of ADK activity, it is reasonable to speculate that ADK serves as a regulator in bone metabolism. Additionally, AMPK signaling, which is literally a downstream metabolic switch of ADK, has been shown to ameliorate osteoporosis associated with T2DM by dampening osteoclast differentiation [81]. This provides an alternative possibility for ADK to participate in bone metabolism. However, there is much less investigation in this regard.

The first piece of evidence of ADK protecting against bone disease came from two decades ago. With an adjuvant arthritis rat model, Boyle et al. suggested that ADK inhibition by ABT-702, a selective and potent ADK inhibitor, exerted chondroprotective effects by restraining NF-kB inflammatory response and ameliorating joint destruction [82]. However, the exact underlying mechanism of action was not clear in this study. A more recent study in this field exhibited that ADK deletion in myeloid cells led to defective bone formation and spontaneous dwarfism *in vivo* and *in vitro* which was associated with increased osteoclastogenesis and bone resorption [83]. The detrimental effect of ADK inhibition in bone growth retardation and excessive bone resorption is contrary to the beneficial effect of ADK inhibitor on improving arthritis. However, this discrepancy could be attributed to animal models from different species, different parameters evaluated, and more importantly the strategies (local or systemic) of ADK

inhibition *in vivo*. More investigations are warranted to further reveal the role of ADK in bone disease and provide a sound explanation for it.

ADK and cancer

Similar to the case in other disorders aforementioned, the role of adenosine and its receptors in cancer have been extensively investigated and well documented. High adenosine levels have been demonstrated in various tumors [84]. Notably, several selective adenosine receptor inhibitors developed by famous pharmaceutical companies have been put into clinical trials for the treatment of cancers [19], the results of which are awaited with much anticipation and excitement. However, in addition to regulating adenosine concentration, ADK possesses immune-modulating and angiogenesis-regulating properties, which are closely related to cancer development. Therefore, the investigation of ADK in cancer treatment has been gaining more attention recently [85, 86].

Hypoxia has been identified as a hallmark of the tumor microenvironment, which favors tumor growth and progression partially through immunosuppression. ADK is a well-known enzyme inhibited by hypoxia, a concept first brought up by Decking and colleagues in 1997 [6]. Their studies with hearts from guinea pigs and mathematical models indicated that ADK metabolized more adenosine in normal hearts than in hypoxic hearts, which was attributed to pronounced inhibition of ADK by hypoxia[6]. Furthermore, Evidence indicated that ADK mRNA level was much higher in human colorectal cancer specimens than normal tissue [87]. However, it is unclear how the alterations of ADK expression contributed to cancer development.

Recently, a study from Boison et al., who is a pioneer and founder researcher investigating the function of ADK in Neural Science, indicated that ADK expression significantly decreased in 7 of 11 patients with either hepatocellular carcinoma (HCC) or intrahepatic cholangiocarcinoma (ICC)[85]. In agreement with this, their transgenic mouse model with ADK reduction in the liver renders the liver more susceptible to the carcinogen diethylnitrosamine (DEN) and the tumor promoter phenobarbital (PB)[85]. Though encouraging, they failed to unravel the mechanism in action. There are two isoforms of ADK identified in humans. The short isoform is cytosolic, whereas the long isoform is localized in the nucleus, which is probably required for maintaining methylation reactions [88]. ADK deletion-induced global hypomethylation, an epigenetic change occurred earliest in the transformation of a normal cell to a malignant cell [89], was probably in charge due to the complete lack of ADK nuclear isoform in their specific mouse model. However, the involvement of adenosine receptor-related effects cannot be ruled out for the cytoplasmic ADK was significantly lower in the mouse model as well.

Understanding the molecular events underlying alterations in cancer cell metabolism is essential to define specific biomarkers and novel treatment targets. We believe that the role of ADK in the behavior of cancer progression warrants further study, and ADK-based intervention holds additional promise in this regard.

Aspects to be exploited: a focus on liver

ADK presents in most tissues with the highest abundance in the liver. Moreover, liver is the site where 85% of transmethylation reactions take place. Therefore, one of the primary phenotypes of whole body ADK-ablation in patients and rodents is liver dysfunction and hepatic encephalopathy [1, 8, 9]. Increased urinary adenosine excretion and unresolved hypermethioninemia were other clinical conditions in those patients [8]. The disrupted transmethylation reactions resulted from ADK deficiency is tentatively speculated to be a mechanism for liver steatosis. Energy depletion as evidenced by the dramatic reduction in AMP, ADP, and ATP in mouse liver homogenates could be another mechanism [1]. However, other effects mediated by AMPK and adenosine receptors cannot be ruled out. While the observation of ADK deficiency in the liver appears paradoxical to our current understanding of beneficial effects of ADK inhibition in other tissue/organ above-mentioned, it is important to highlight that ADK plays a critical role in liver physiology. Liver-specific ADK KO animal models are warranted to model and understand the liver phenotype of the human ADK mutation.

Consideration for the clinic

Current evidence on how ADK inhibition affects the outcomes of various diseases like CVD, DM, etc. is inconclusive; however, given the plural functions of ADK in cellular metabolism, ADK-oriented therapeutics may impart better effects than some proposed combination strategies. This rapidly growing field is not new. Indeed the investigation of ADK biochemistry can be traced back to decades ago [90]. The ADK-

based treatments for the neural disease have been extensively covered elsewhere in the literature and are thus largely omitted here. Due to druggability issues [91], the translation of the abundant knowledge of ADK pathophysiology to clinical application has been slow.

Manipulating the adenosine receptor signaling

Clearly, one important part of ADK function is through regulating adenosine signaling via the four adenosine receptors. Adenosine *per se* and its receptors have long been targets for drug development [19, 92]. However, the clinical application of adenosine is restricted to first aid for supraventricular tachycardia due to its extremely short half-life and potential cytotoxicity [93]. Despite a large amount of selective adenosine receptor ligands reported in the literature, only one (Lexiscan; Astellas Pharma), which is an adenosine $A_{2A}R$ agonist, has been approved by the US Food and Drug Administration (FDA) for use as a pharmacologic stress agent [94]. The majority of adenosine receptor ligands fail to exhibit a beneficial therapeutic index due to systemic side effects [95]. Targeting ADK to augment extracellular adenosine levels might be a therapeutic alternative since the side effect profile of ADK inhibitors has been reported to be improved compared to direct adenosine receptor ligands [10].

Nutritional strategies

So far, 21 human patients with ADK genetic deficiency have been reported. Methionine-restricted diet (17–20 mg/kg/day) [11] was recommended to improve symptoms, the liver phenotype in particular, in these patients. It seems reasonable that

reduced exogenous methionine would partially release the load of abnormities of adenosine metabolism and to some extent rescue the transmethylation reactions. A potential concern of this recommendation, however, is that discontinuation of the diet would lead to acute liver dysfunction [8].

Another dietary treatment strategy to be considered is the ketogenic diet, which is commonly applied in the clinical setting for patients with epilepsy [96-98]. Research shows that ketogenic diet can suppress the expression of ADK [99, 100], which has been proposed to impart beneficial effects for various disorders as outlined above in an adenosine receptor-dependent or -independent manner. Since liver is the major place for ketogenesis, this dietary intervention might display therapeutic effects on liver-related diseases, such as the fatty liver disease, which consumption warrants more investigation in the future.

Conclusion

ADK can regulate all the adenosine signaling synergistically, the capacity to influence global methylation status through altering the transmethylation reactions, and the possibilities to affect AMPK activity and energy homeostasis. It is the hub of plenty of metabolic networks and thus its dysfunction is closely related to various metabolic diseases. Thanks to the advance of gene editing technologies like the Cre-LoxP system, researches could gain an insight into the functions of ADK in individual tissue/organ. Although conclusive evidence of ADK dysfunction leading to metabolic diseases is lacking, ADK inhibition has been repeatedly reported to be beneficial for CVD and DM.

More studies are warranted to unveil the ADK pathophysiology in liver disease. This rapidly growing field is not new, and ADK-based therapies have been investigated in both academia and industry. Notably, the potential applications of ADK inhibition may be limited due to an unacceptable risk profile in off-target tissues, such as the brain. Nevertheless, ADK-oriented therapeutics might open a new chapter in the study of metabolic disease prevention and treatment.

CHAPTER III

THE RELEVANCE OF HEPATIC ADK IN NUTRIENT HOMEOSTASIS UNDER DIFFERENT NUTRITIONAL CONDITIONS.

Introduction

Physiologically, the concentration of adenosine is 30-200nM, while it is up to 1-30μM upon the challenge of acute or chronic inflammation to fulfill immunosuppressive effects mainly by activation of certain types of adenosine receptors on the cell membrane[28]. Adenosine comes from the dephosphorylation of extracellular ATP via CD39 and CD73[101], from the dephosphorylation of intracellular AMP by 5'-nucleotidase as well as the conversion of intracellular S-adenosyl homocysteine (SAH) from the methionine cycle [10, 102]. Adenosine can be transported in and out of cells dynamically via equilibrative nucleoside transporters (ENTs). Extracellular adenosine signals through adenosine receptors and thus participates in a series of downstream events. In order to maintain methylation reactions, intracellular adenosine is constantly removed. The major routes of adenosine removal are either deamination to form inosine via adenosine deaminase (ADA) or phosphorylation to AMP via ADK [10, 34]. Since the K_m of ADK for adenosine is much lower than that of ADA, ADK is the principal enzyme responsible for regulating the concentration of adenosine. There are two isoforms of ADK identified in humans [103]. The short isoform is cytosolic, whereas the long isoform is localized in the nucleus, which is probably required for maintaining methylation reactions [103].

By regulating the concentration of adenosine and impacting multiple pathways via adenosine signaling modulation and epigenetic reprogramming, ADK has been identified as an upstream regulator for complicated metabolic networks. Boison and co-authors performed intensive studies with transgenic mice either overexpressing or disrupting ADK in the neuron, which caused either greatly enhanced or reduced occurrence of epileptic seizures [104]. The same group also confirmed that ADK mutant mice died in the early days of postnatal and that such early postnatal lethality resulted from liver failure [1]. Therefore, multiple lines of evidence indicate that ADK might be required in normal liver function; its overexpression, however, is not necessarily beneficial, and could even be detrimental. Moreover, ADK is widely expressed in many tissues with the highest abundance in the liver, which is the major place for nutrient metabolism. Liver is the site where 85% of transmethylation reactions take place. In addition to neural symptoms like epilepsy, liver dysfunction is another manifestation of ADK mutant in both human patients and animal models. All of these clues suggest that hepatic ADK might participate in the regulation of nutrient homeostasis. To date, however, there is no direct evidence showing the relevance of hepatic ADK in nutrient homeostasis under different nutritional conditions. Therefore, we propose to determine the relevance of hepatic ADK in glucose and lipid metabolism by comparing the hepatic ADK levels and the accompanied phenotypes of wild type mice fed with a high-fat diet (HFD) compared to mice fed with a low-fat diet (LFD).

Materials and Methods

Animal experiments

Five-to-six-week old male C57BL/6J wild type (WT) mice purchased from The Jackson Laboratory were fed with an LFD (Research Diets, #D12450B) or an HFD (Research Diets, #D12492) for 12 weeks (n=6-8 per group). The LFD consists of 10.4% fat, 69.1% carbohydrate and 20.5% protein of total kcal, while the HFD is made up of 60.3% fat, 21.3% carbohydrate and 18.4% protein of total kcal [105]. During the feeding regimen, body weight and food intake of mice were monitored weekly. At the last two weeks of feeding regimen, mice were subjected to glucose and insulin tolerance test separately, as specified in detail below. At the end of the animal study, mice were anesthetized, and the abdominal cavities were opened carefully. Blood was drawn directly from the heart through the diaphragm, and plasma was further obtained by centrifugation at 3000rpm for 15 min at 4°C. Tissue samples, including liver and fats, were removed, weighed and snap-frozen immediately in liquid nitrogen and then stored in -80 °C for further analysis. Part of the liver tissues were fixed and embedded for histological analyses (H&E staining) as described below.

All animal experiments and care were approved by the IACUC (Institutional Animal Care and Use Committee) at Texas A&M University (College Station, TX) under National Institutes of Health guidelines.

Glucose and insulin tolerance test

Glucose tolerance test (GTT) is widely employed to assess the body's capability to cope with an i.p. injected glucose load. The insulin tolerance test (ITT) is usually used in

conjunction with GTT, to determine systemic insulin sensitivity after an i.p. injection of insulin. Mice fasted for 4 h and were then injected with a single dose of D-glucose (2 g/kg BW, 0.1 mL/10g BW) or insulin (1 U/kg BW, 0.1 mL/10g BW), respectively. For GTT, blood samples were collected through the tail vein before glucose injection and at 30, 60, 90 and 120 min afterward. Similarly, for ITT, blood samples were collected through the tail vein before insulin injection and at 15, 30, 45 and 60 min after injection. The plasma glucose concentrations were determined by a glucose assay kit (Sigma, St. Louis, MO).

Histological analysis of liver tissues

Livers from mice fed with an LFD or an HFD were fixed in 10% neutral buffered formalin for >24 h, dehydrated, and then embedded in paraffin. Sections of 5 μM thickness were stained with hematoxylin and eosin (H&E) for morphologic studies under a light microscope.

Hepatic cytokine determination

TNFα and IL-6 concentrations in the liver were quantified by commercially available enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Tissue samples of both groups were analyzed together in the same analytic batch.

Western blot assays

Around 50mg frozen-liver tissues were homogenized and lysed in RIPA buffer (Millipore, Temecula, CA, USA) containing Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) and 1 mM PMSF. The Bicinchoninic acid

(BCA) Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) was used to quantify the concentrations of the protein samples. Fifty ugs of total protein was electrophoresed through a 10% SDS polyacrylamide gel and subsequently transferred to 0.45 mm polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline (TBS) buffer for 2h at room temperature. Rabbit monoclonal antibodies against ADK (Abcam, Cambridge, MA, USA; cat no. ab38010; 1:1000), p- NFκB / NFκB (Cell Signaling Technology, Danvers, MA, United States; cat no. 3033s/8242s; 1:1000), pJNK/JNK(Cell Signaling Technology, Danvers, MA, USA; cat no.9251L/9252L; 1:1000), and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA; cat. no. sc-25778; 1:1000) were used to detect protein levels in liver tissue lysates. The membranes were incubated with the primary antibodies in 1% bovine serum albumin (Sigma Aldrich; Merck KGaA, Germany; cat no. 12659) overnight at 4˚C and washed three times with Tris-buffered saline-Tween (TBST). Subsequently, membranes were incubated with horseradish peroxidase-conjugated secondary goat anti rabbit antibody (Invitrogen Corporation, Frederick, USA; cat. no. 31460; 1:10,000) in TBST for 1 h at room temperature and washed three times with TBST again. Membranes were visualized using Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific; Frederick, Maryland, USA; cat no. 32106). Bands were quantified using Image Lab 5.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Statistical analysis

Statistical analyses were performed with GraphPad Prism 6.01 (GraphPad Software, Inc., La Jolla, CA, USA). Data were presented as mean ± SEM. Two-tailed unpaired t-tests were used for comparisons between two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

First, we assessed the dynamic changes in body weight for the diet. HFD feeding caused more body weight gain compared with LFD feeding, which reached a statistical significance since week 4 (Fig. 2A). Differences in adipose tissue weights between diets were also evaluated. In comparison with LFD, HFD significantly increased adipose tissue masses (Fig. 2B). The effects of HFD in C57 BL/6 mice on glucose tolerance and insulin tolerance *in vivo* were assessed by GTT and ITT, respectively (Fig. 2C and D). Fat-feeding induced robust glucose intolerance in this mouse line, as reflected by sustained hyperglycemia throughout the GTT in HFD-fed mice when compared to LFD-fed mice (Fig. 2C). Similarly, the plasma glucose levels in HFD mice didn't tend to drop as much in contrast to those in LFD mice (Fig. 2D), indicating an insulin resistance in the HFD mice.

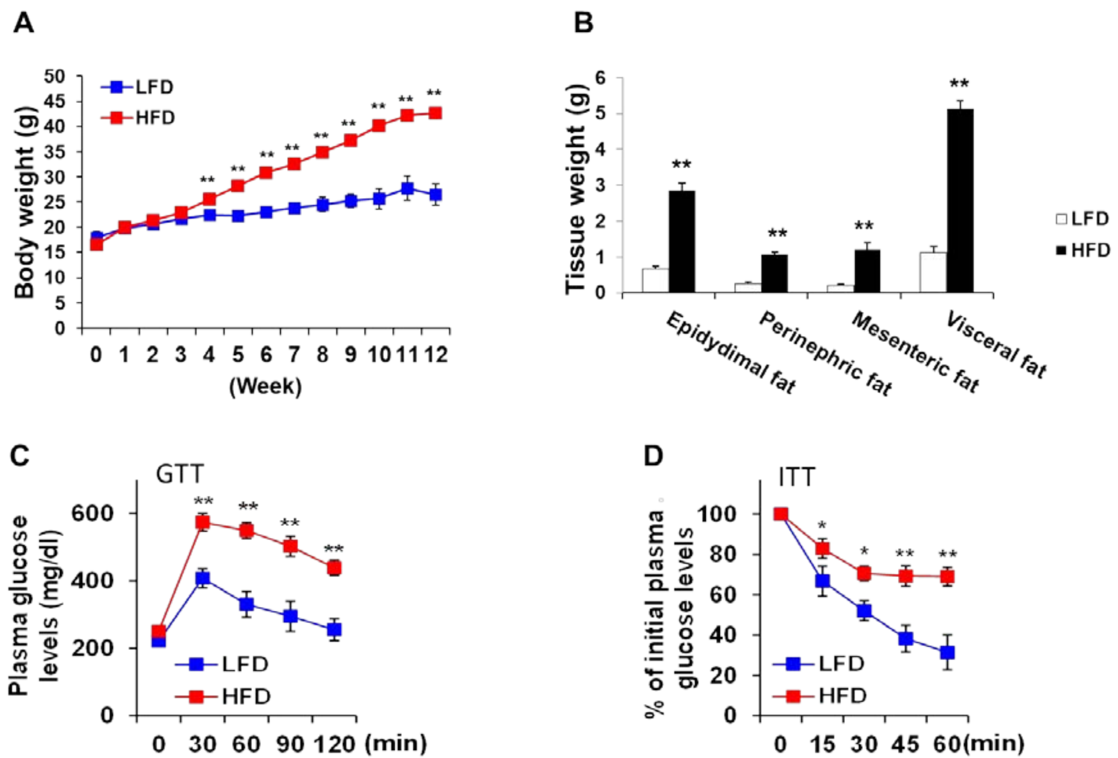

Figure 2: HFD induces obesity and insulin resistance.
Five-to-six-week old male C57BL/6J wild-type mice were fed with an LFD or an HFD for 12 weeks. Data are presented as mean ± SEM, n= 6-8. (A) Body weight was monitored weekly during the feeding period. (B) Tissue weight of different white adipose tissue depots. Visceral fat = Epididymal fat + Perinephric fat + Mesenteric fat. (C) Glucose tolerance test (GTT). (D) Insulin tolerance test (ITT). For C and D, mice fasted for 4 h and were then injected with a single dose of D-glucose (2 g/kg BW, 0.1 mL/10g BW) or insulin (1 U/kg BW, 0.1 mL/10g BW), and plasma glucose levels were determined at the indicated time points. *, P<0.05 and **, P<0.01, HFD vs LFD.

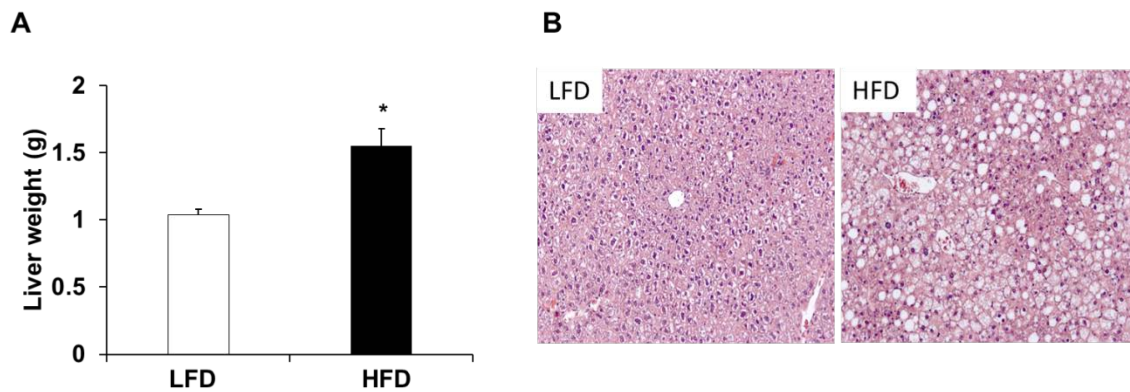

Figure 3: HFD induces hepatic fat deposition.
Five-to-six-week old male C57BL/6J wild-type mice were fed with an LFD or an HFD for 12 weeks. Data are presented as mean ± SEM, n= 6-8. (A) Liver weight. (B) H&E staining of liver sections. *, $P<0.05$ and **, $P<0.01$, HFD vs LFD.

To further characterize liver phenotypes, we analyzed liver net weight and histology data. Overall, liver weights were higher in HFD mice compared to LFD mice (Fig. 3A). Moreover, HFD mice developed hepatic steatosis, as evidenced by the accumulation of excessive fat droplets in the liver (Fig. 3B).

Furthermore, we examined liver inflammation, another classic phenotype of chronic HFD feeding. As expected, we observed increased phosphorylation of NFκB and JNK in the liver (Fig. 4A), suggesting the activation of the two pro-inflammatory signaling pathways. Moreover, HFD treatment induced a significant increase of pro-inflammatory cytokine secretion in the liver tissues (Fig. 4B).

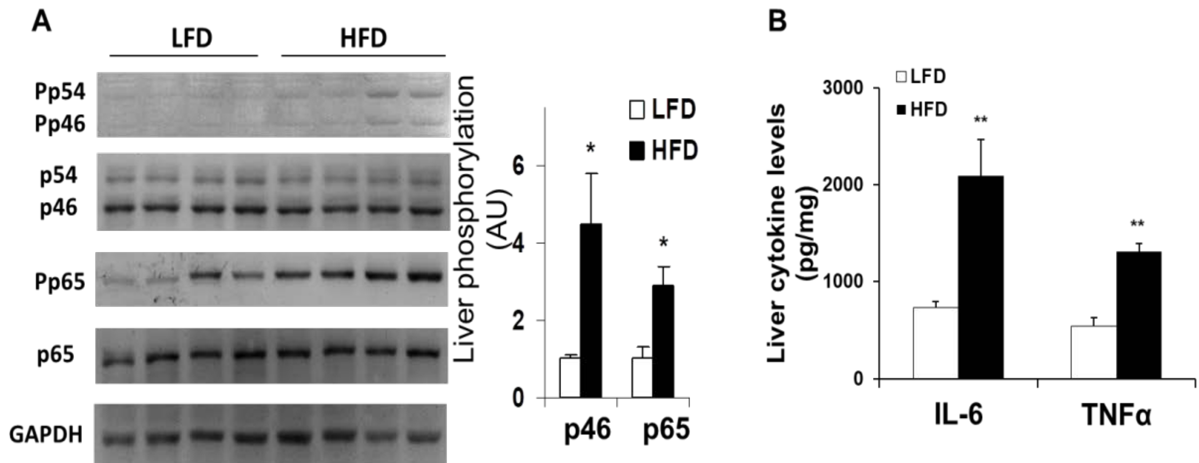

Figure 4: HFD induces hepatic metabolic inflammation.
Five-to-six-week old male C57BL/6J wild-type mice were fed with an LFD or an HFD for 12 weeks. Data are presented as mean ± SEM, n= 6-8. (A) Hepatic inflammatory signaling determined by western blot analysis and quantified with densitometry. (AU, arbitrary unit). (B) Hepatic pro-inflammatory cytokine levels determined by ELISA analysis. *, P<0.05 and **, P<0.01, HFD vs LFD.

To evaluate protein expression levels of ADK in HFD vs LFD, western blot analysis was performed. Hepatic ADK protein levels were much higher in HFD mice compared with that in LFD mice (Fig. 5), which indicates ADK got involved in HFD-induced NAFLD.

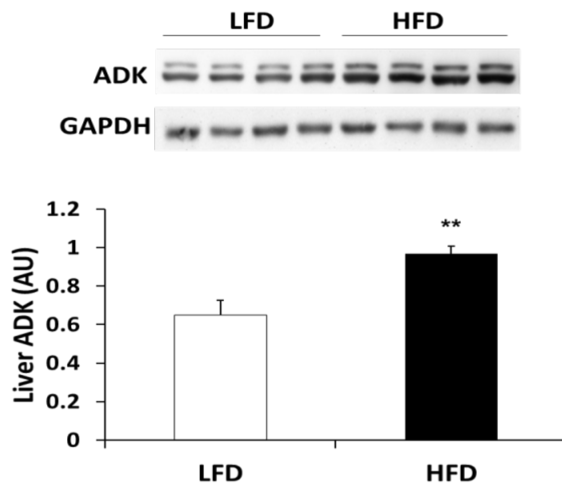

Figure 5: HFD upregulates hepatic ADK expression.
Five-to-six-week old male C57BL/6J wild-type mice were fed with an LFD or an HFD for 12 weeks. Hepatic ADK protein levels determined by western blot analysis and quantified with densitometry. (AU, arbitrary unit). *, P<0.05 and **, P<0.01, HFD vs LFD.

Overall, as reported repeatedly, chronic HFD feeding predisposed C57 BL/6 mice to obesity, insulin resistance, fatty liver, and low-grade inflammation. Our data provided confirmatory evidence of the ADK dysfunction in response to the challenge of excess energy supply.

Discussion

Our observations were consistent with the classic HFD feeding phenotypes including obesity, insulin resistance, fatty liver, and inflammation [106]. For the first time, we were able to confirm that hepatic ADK was upregulated upon HFD feeding, which lays the foundation of our hypothesis.

Obesity and insulin resistance are usually brought about by an affluent nutrient condition attributed to an imbalance between energy intake and energy expenditure [107]. Additionally, NAFLD generally coexists with dyslipidemia, obesity, and insulin resistance [108]. Therefore, HFD-induced NAFLD is a classic animal model for research on fatty liver disease. It has been well established that insulin resistance, excessive fat deposition, and low-grade chronic inflammation, as well as the vicious circle among them, are major mechanisms of obesity-related NAFLD [109]. To date, there is no direct evidence showing the involvement of hepatic ADK in the initiation and progression of diet-induced NAFLD, however, ADK-based interventions have been investigated in preclinical studies in many areas including inflammation [110], which is one of the "hits" for NAFLD. It is reported that ADK deficiency in endothelial cells exhibited an anti-inflammatory effect through decreasing the levels of methylated histone 3 on lysine 4 (H3K4) [12]; H3K4 could transcriptionally activate gene expression for vascular inflammation. Additionally, Pye et al. reported a reno-protective effect of ADK inhibition, which is likely mediated by attenuating the oxidative stress and inflammation, in streptozotocin-induced diabetic mice [78]. More importantly, Xu et al. suggested that ADK deficiency in endothelial cells protected against HFD-induced insulin resistance in mice, which was associated with increased nitric oxide production [111]. Taken together, ADK inhibition might play a multifaceted protective effect on aspects of diet-induced NAFLD, including, but not limited to, inflammation and insulin resistance.

ADK is likely involved in diet-induced NAFLD. However, with limited clues here, it remains unclear whether the higher hepatic ADK levels contributed to the progression of

NAFLD, or they were simply a secondary effect. To further address this issue, we employed liver-specific ADK KO mouse models for the following investigations.

CHAPTER IV

LIVER-SPECIFIC ADK KNOCKOUT PROTECTS AGAINST HFD-INDUCED NAFLD.

Introduction

NAFLD is characterized by fat deposition in hepatocytes due to causes other than excessive alcohol consumption [112]. NAFLD represents a wide spectrum of disease ranging from reversible steatosis, steatohepatitis to irreversible cirrhosis[113]. The current prevalence of NAFLD varies from 9 to 37% worldwide with an average estimated to be 24%[114], and it predisposes one to liver-related complications like cirrhosis, hepatocellular carcinoma and extrahepatic disease such as cardiovascular disease (CVD)[115]. NAFLD is currently the second-leading etiology for liver transplantation in the US [116]. Accordingly, it is a heavy economic burden worldwide. In the US alone, the annual cost of NAFLD is about a hundred billion dollars and it is steadily increasing [114]. Lifestyle modification is the most important and fundamental method to solve this global health problem, while several factors complicate it [117]. Medications like insulin sensitizers, vitamin E, and omega-3 fatty acids have proven some histological improvements in NASH but clinical outcomes are still not satisfying[118]. Current medications mainly aim to manage comorbidities and to date no FDA-approved medications available for NAFLD. Therefore, it would be of particular importance in developing novel and effective treatments for NAFLD.

Without a better understanding of the pathogenesis of NAFLD, we will never be able to treat it effectively or precisely, without, or at least with limited, unwanted or off-target effects. While still a controversial topic, the classic model to explain the pathogenesis of NAFLD is a two-hit hypothesis[113]. Briefly, the first hit is excessive fat accumulation in the liver, which could result from increased de novo lipogenesis, decreased fatty acid oxidation, decreased VLDL output, etc. The second hit is the initiation and propagation of chronic low-grade inflammation, which could come from activation of macrophage toll-like receptors by saturated fatty acids, oxidative stress elicited by mitochondria dysfunction, and hepatocyte necrosis due to lipotoxicity [109]. However, it becomes rapidly evident that this view is too simplistic. Consequently, a multiple-hit hypothesis has substituted the two-hit hypothesis[119]. Nowadays, it's relatively convincing that fat deposition, inflammation, and insulin resistance, as well as their interplay, contribute to the pathogenesis of NAFLD[109]. Therefore, we are particularly interested in strategies protective of these aspects.

Chronic low-grade inflammation has been demonstrated to contribute to many metabolic diseases including NAFLD [120]. Abnormal fat deposition is the manifest of NAFLD. Insulin resistance indicates an impaired capability of insulin to inhibit lipolysis while the capacity of insulin to promote lipogenesis is reserved [121]. These three aspects of NAFLD form a vicious cycle, and the deterioration of any aspect would drive the cycle forward and make the phenotypes of NAFLD worse[109]. Adenosine mainly gets involved in glucose and lipid metabolism through adenosine signaling mediated by adenosine receptors [16], or through epigenetic regulation mediated by transmethylation

reactions [122]. As an upstream regulator of adenosine homeostasis, hepatic ADK could possibly participate in the pathophysiologic process of NAFLD.

ADK is an evolutionally conserved enzyme with multiple functions. However, our environment has changed considerably in the past decades, especially in regards to what we eat. Nutrition transition [123], from food deficiency to food affluence, and from low energy density to high energy density, could be an inducer for the dysregulation of ADK. The increased incidence of NAFLD is also somehow the product of nutrition transition [124, 125]. Since our preliminary data indicate that ADK protein expression was responsive to different nutritional conditions, it would be of great interest to see if ADK dysfunction promotes the happening of NAFLD. Therefore, we aimed at examining the influences of hepatic ADK deficiency on metabolic consequences of HFD-induced NAFLD by the employment of two conditional liver-specific ADK knockout mouse models.

Materials and Methods

Mouse generation and breeding

ADK floxed ($ADK^{F/F}$) mice were generated by insertion of loxP sites on both sides of exon 7 of the ADK gene. $ADK^{F/F}$ mice were then mated with Alb-cre mice—a mouse line in which Cre is specifically expressed in albumin expressing hepatocytes (stock No. 003574; The Jackson Laboratory, Bar Harbor, ME). The resulting Albcre-$ADK^{F/+}$ mice were ADK partially deficient selectively in hepatocytes. Male AlbCre-$ADK^{F/+}$ mice were further crossed with female AlbCre-$ADK^{F/+}$mice, and liver-specific ADK-disrupted

homozygous AlbCre-$ADK^{F/F}$ mice, heterozygous AlbCre-$ADK^{F/+}$ mice, and AlbCre-$ADK^{+/+}$ control mice, as well as $AlbCre^{-}$ control ($AlbCre^{-}$-$ADK^{F/F}$, $ADK^{F/+}$, and $ADK^{+/+}$) mice were generated and identified. Because homozygous mice couldn't survive more than 4 weeks, we harvested live tissues from 3-week old homozygous mice for analysis. All mice were provided with water ad libitum and maintained on a 12:12-hour light:dark cycle in a specific pathogen-free facility at 22 ± 1 °C. All animal experiments and care were approved by the IACUC (Institutional Animal Care and Use Committee) at Texas A&M University (College Station, TX) per National Institutes of Health guidelines.

Confirmation of liver-specific ADK disruption.

The genotype of liver-specific ADK-disrupted homozygous, heterozygous as well as control mice were identified by PCR-based genotyping performed on tail-extracted genomic DNA at wean and were retrospectively confirmed with western blot analysis in experimental animals.

Animal studies

Five-to-six-week old male and female liver-specific ADK-disrupted heterozygous knockout and control mice (n=6-8 per group) were fed with an HFD for 12 weeks. During the 12-week feeding period, body weight and food intake were monitored weekly. GTT and ITT were performed in the same way as described in Chapter III. After the feeding regimen, mice were anesthetized, blood samples were collected and plasma obtained by centrifugation at 3000rpm for 15 min at 4°C. Tissue samples were removed, weighed and snap-frozen immediately in liquid nitrogen and then stored in -80 °C for further analysis.

Additionally, twelve-to-sixteen-week old male ADKF/F mice were tail vein injected with the pAAV TBG FFLuc which is an adenovirus with fluorescence signaling that expresses Cre under control of the liver-specific TBG promoter at a concentration of 1.3*108 pfu/g mice to generate an alternative ADK-disrupted mouse model, denoted as TBG virus. pAAV TBG FFLuc was a gift from Phillip Zamore (Addgene plasmid # 35658). Another set of gender-, age-, and genotype-matched mice were injected with control virus as control, denoted as Ctrl virus. These mice were fed with chow diet and harvested around 18 days after injection within the window of no visible phenotype at 2 weeks and mouse mortality at 3 weeks.

The effects of ADK disruption on aspects of obesity-associated NAFLD

Liver H&E staining and the inflammatory markers were accessed in a similar way to Chapter III. For the virus-mediated knockout mouse model study, hepatic ketone bodies' concentrations were determined by commercially available kits (BioAssay Systems; Hayward, CA, USA).

RNA isolation, reverse transcription, and real-time PCR.

Total RNA was extracted from RAN ladder preserved liver tissues using RNA STAT-60 (Thermo Fisher Scientific; Frederick, Maryland, USA) and cDNA was generated using the GoScript™ RT reagent kit (Bio-Rad, Hercules, CA, USA) at 37˚C for 30 min and 85˚C for 1 min according to the manufacturer's protocol. RT-qPCR was performed using the SYBR Green PCR kit on a Mastercycle (LightCycler® 480 system; Roche). All primers were provided by Invitrogen (Thermo Fisher Scientific; Frederick, Maryland, USA) and were available as request. The mRNA levels were analyzed for

acetyl CoA carboxylase 1 (ACC1), fatty acid synthase (FAS), and carnitine palmitoyltransferase 1 (CPT1) in liver tissue samples. PCR products were assessed by dissociation curve and gel electrophoresis, results were normalized to 18s ribosomal RNA. Normalized data were then analyzed using the $2^{-\Delta\Delta Ct}$ method [126].

Statistical analysis

Statistical analyses were performed with GraphPad Prism 6.01 (GraphPad Software, Inc., La Jolla, CA, USA). Data were presented as the mean ± SEM. We performed statistical analysis using one-way analysis of variance (ANOVA) to determine the differences between groups followed by the Dunnett’s post-hoc analysis. Two-tailed unpaired t-tests were used for comparisons between two groups where appropriate. The cutoff for statistical significance was set at a $P<0.05$.

Results

The liver-specific ADK knockout mouse model was successfully generated with Cre-LoxP technology as we can see in Fig. 6A. Because homozygous mice cannot survive more than 4 weeks, we harvested live tissues and analyzed ADK levels when the mice were around 3 weeks old. The ADK protein levels in heterozygous mice significantly decreased and were even less in homozygous mice compared with control mice, suggesting a “gene dose”-dependent knockout effect. We also retrospectively confirmed an ADK partially KO in experimental heterozygous animals (Fig. 6B).

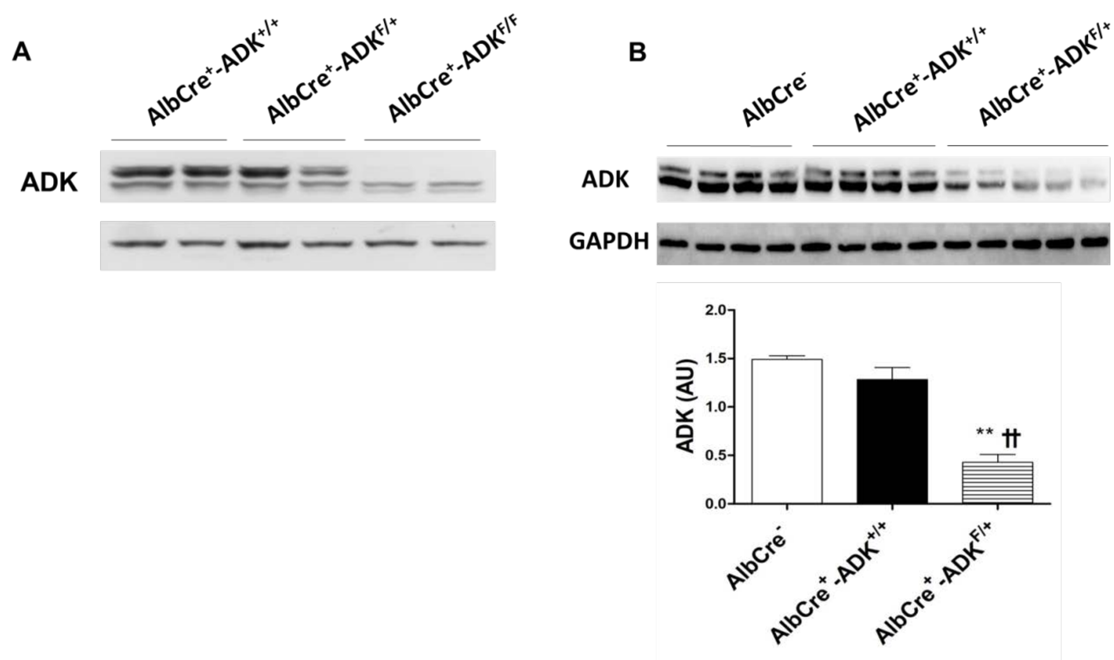

Figure 6: The confirmation of hepatic ADK knockout with Cre-LoxP technology. (A) Liver tissues from around 3-week-old homozygous, heterozygous, and control mice were harvested for western blot analysis for ADK levels. (B) Liver tissues from experimental mice were retrospectively examined for ADK levels with western blot analysis. (AU, arbitrary unit). *, P<0.05 and **, P<0.01, Albcre-ADK$^{F/+}$ vs. AlbCre-; †, P<0.05 and ††, P<0.01, Albcre-ADK$^{F/+}$ vs. Albcre^{+}-ADK$^{+/+}$.

High-fat mice of both gender with liver-specific ADK deficiency exhibited improved glucose tolerance when compared with ADK intact mice because the blood glucose concentrations were lower throughout the time course of GTT (Fig. 7A and B). Accordingly, these mice showed ameliorated insulin resistance as well, since the glucose levels decreased much more in both male and female heterozygous mice compared to the respective controls (Fig. 7C and D). The hepatic fat deposition was greatly improved in heterozygous mice of both genders. As shown in Fig. 8, small amounts of lipid droplets

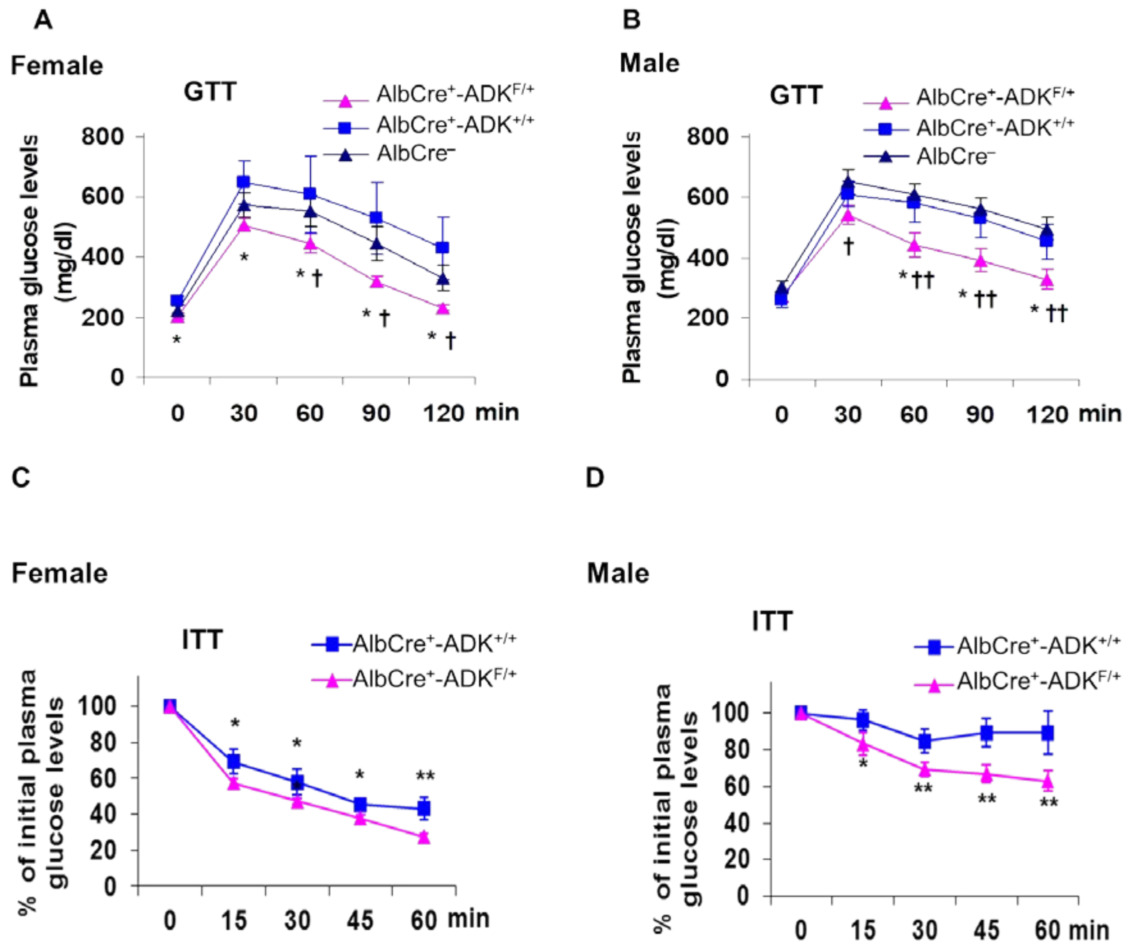

Figure 7: Hepatocyte ADK regulates systemic glucose homeostasis.
Five-to-six-week old male and female heterozygous and control mice were fed with an LFD or an HFD for 12 weeks. Data were presented as the mean ± SEM, n= 6-8. (A) Glucose tolerance test (GTT) for female mice. (B) Glucose tolerance test (GTT) for male mice. (C) Insulin tolerance test (ITT) for female mice. (D) Insulin tolerance test (ITT) for male mice. For GTT and ITT, mice fasted for 4 h and were injected with a single dose of D-glucose (2 g/kg BW, 0.1 mL/10g BW) or insulin (1 U/kg BW, 0.1 mL/10g BW), respectively, and plasma glucose levels were determined at the indicated time points. *, $P<0.05$ and **, $P<0.01$, Albcre-ADK$^{F/+}$ vs. Albcre^{+}-ADK$^{+/+}$; †, $P<0.05$ and ††, $P<0.01$, Albcre-ADK$^{F/+}$ vs. AlbCre^{-}.

were found in mouse liver sections of heterozygous mice of both genders after 12 weeks on HFD. In contrast, WT mice presented with severe hepatosteatosis, including massive accumulation of large lipid droplets. The fat deposition was more severe in male mice than female mice, which might have something to do with the different sex hormone levels [127]. Moreover, the inflammatory response in liver tissues of heterozygous mice got significantly improved (Fig. 9).

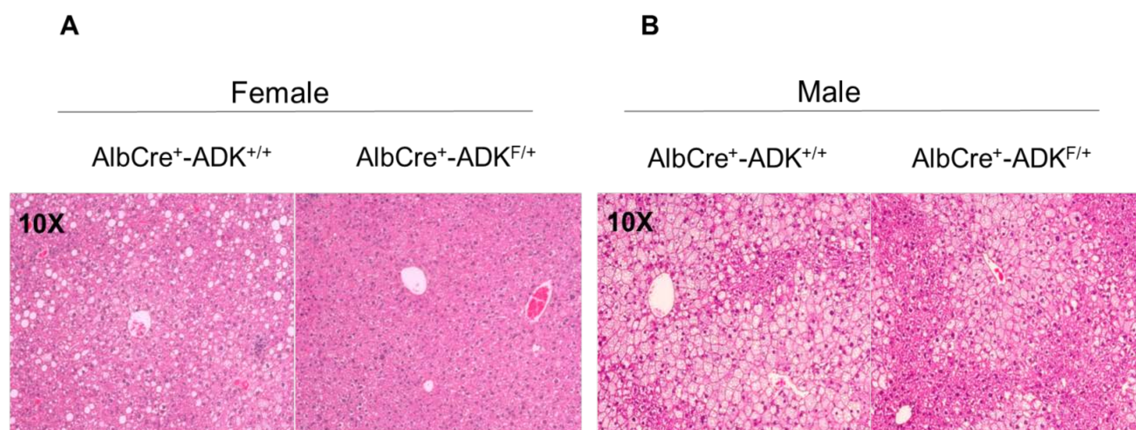

Figure 8: Hepatocyte ADK regulates hepatic fat deposition.
Five-to-six-week old male and female heterozygous and control mice were fed with an LFD or an HFD for 12 weeks. H&E staining of liver sections.

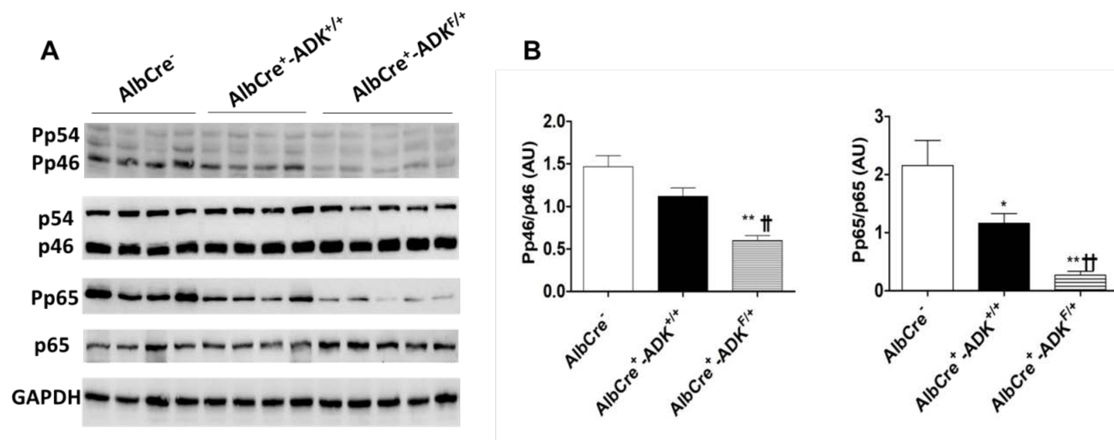

Figure 9: Hepatocyte ADK regulates hepatic inflammation.
Five-to-six-week old male and female heterozygous and control mice were fed with an LFD or an HFD for 12 weeks. (A) Liver inflammatory responses indicated by western blot analysis. (B) Quantification of western blots with Image J using densitometry and normalized to GAPDH. (AU, arbitrary unit).

Collectively, these data suggested a protective role of hepatic ADK KO in HFD-induced NAFLD. However, the ADK gene function was lost during pre-natal and post-natal development in this animal model which might trigger some unexpected compensatory consequences [128]. Therefore we used an adeno-associated virus (AAV) mediated KO mouse model to address this problem. This mouse model was generated in a similar way to Cre-loxP system except that we delivered the Cre recombinase carried by the AAVs via a tail vein injection ($1.3*10^8$ pfu/g mice) into adult $ADK^{F/F}$ mice. These animals can undergo normal embryonic and postnatal development until the time of gene inactivation. We didn’t notice any gross physiological abnormalities 2 weeks

after injection, however, almost all TBG virus treated mice died in 3 weeks. Therefore we determined to harvest mice at Day 18 after virus injection.

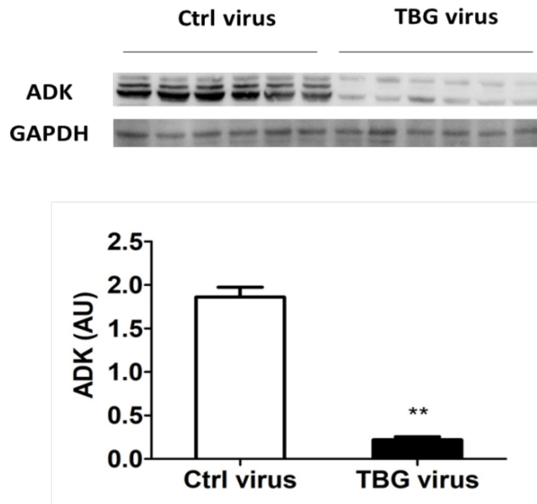

Figure 10: The confirmation of hepatic ADK knockout with TBG virus treatment. This mouse model was generated by delivering the AAV carried liver-specific TBG promotor-driven Cre recombinase or empty AAV via a tail vein injection into the adult $ADK^{F/F}$ mice. Mice were harvested at Day 18 after AAV injection. Liver tissues from experimental mice were retrospectively examined for ADK levels with western blot analysis. (AU, arbitrary unit). *, P<0.05 and **, P<0.01, TBG virus vs. Ctrl virus.

First things first, we confirmed that the hepatic ADK was effectively knockout in TBG virus group compared to control group with western blot analysis (Fig. 10). Before treatment, the body weights of both groups were similar (Fig. 11A). However, the ADK knockout led to an acute weight loss (Fig. 11A), accompanied by visceral fat mass reductions (Fig. 11B). Paradoxically, the liver weight increased in the KO mice (Fig. 12A). Interestingly, liver ketone bodies acetone, acetoacetic acid, and beta-

hydroxybutyric acid (BOH) were 3-5 times higher in the virus-mediated ADK-KO mice in contrast to the levels in control mice (Fig. 12B). We also quantified the mRNA concentrations of ACC1, FAS, and CPT1 in liver tissues (Fig. 12C). The lipogenic genes ACC1 and FAS mRNA concentrations were decreased in the knockout mice when compared with control mice. However, the lipolysis gene CPT1 mRNA level was increased in the knockout mice. Not surprisingly, the inflammatory responses in liver tissues of TBG virus group also greatly dampened (Fig. 13). This was in agreement with the result from the Cre-LoxP conditional ADK KO mouse model.

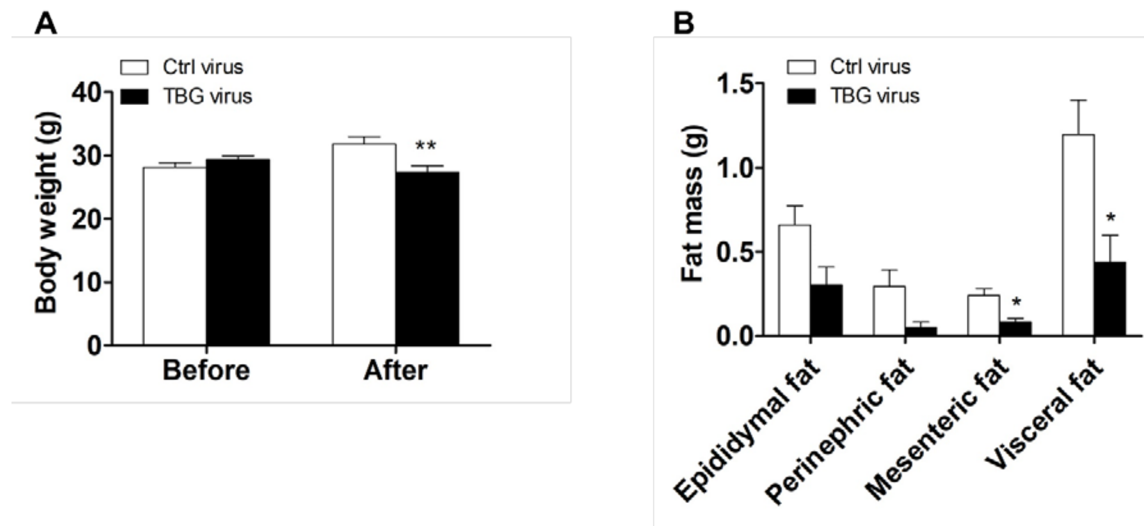

Figure 11: Hepatocyte ADK knockout results in weight loss.
This mouse model was generated by delivering the AAV carried liver-specific TBG promotor-driven Cre recombinase or empty AAV via a tail vein injection into the adult $ADK^{F/F}$ mice. Mice were harvested at Day 18 after AAV injection. Data were presented as the mean ± SEM, n= 6-8. (A) Body weight before tail vein injection and when harvest. (B) Tissue weight of different white adipose tissue depots. Visceral fat= Epididymal fat + Perinephric fat + Mesenteric fat. *, P<0.05 and **, P<0.01, TBG virus vs. Ctrl virus.

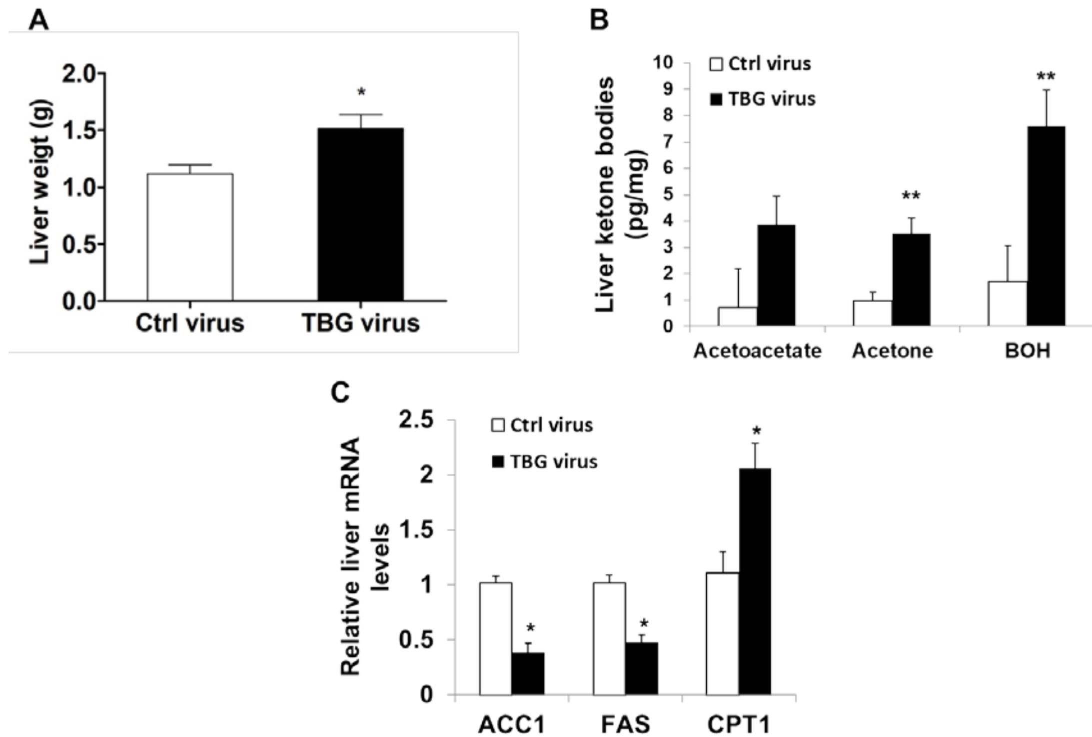

Figure 12: Hepatocyte ADK knockout increases ketogenesis.
This mouse model was generated by delivering the AAV carried liver-specific TBG promotor-driven Cre recombinase or empty AAV via a tail vein injection into the adult $ADK^{F/F}$ mice. Mice were harvested at Day 18 after AAV injection. Data were presented as the mean ± SEM, n= 6-8. (A) Liver weight. (B) Concentrations of ketone bodies in the liver. (C) Liver mRNA levels of genes encoded for key lipid metabolic enzymes were analyzed using real-time PCR. *, $P<0.05$ and **, $P<0.01$, TBG virus vs. Ctrl virus.

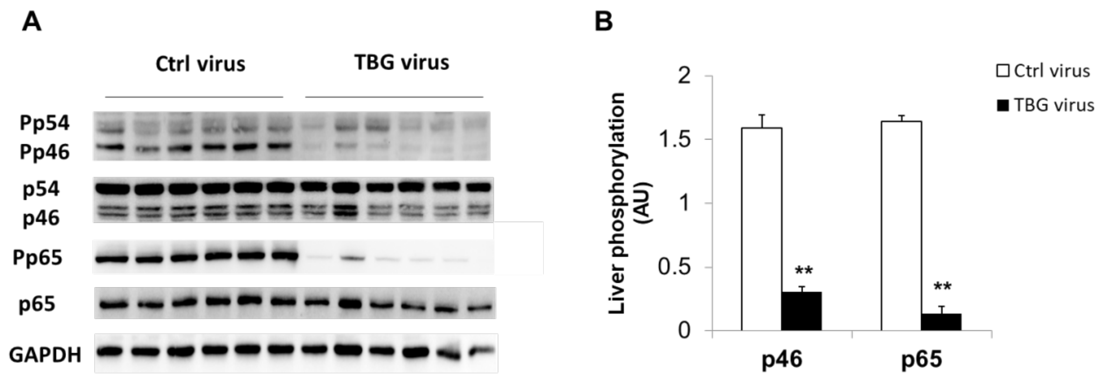

Figure 13: Hepatocyte ADK knockout attenuates hepatic inflammation.
This mouse model was generated by delivering the AAV carried liver-specific TBG promotor-driven Cre recombinase or empty AAV via a tail vein injection into the adult $ADK^{F/F}$ mice. Mice were harvested at Day 18 after AAV injection. (A) Liver inflammatory responses indicated by western blot analysis. (B) Quantification of western blots with Image J using densitometry and normalized to GAPDH. (AU, arbitrary unit). *, P<0.05 and **, P<0.01, TBG virus vs. Ctrl virus.

Discussion

With the Cre-LoxP system-generated conditional ADK KO mouse model, we observed a clear amelioration in aspects of HFD-induced NAFLD, including glucose intolerance, insulin resistance, hepatic steatosis, and liver inflammation. These robust phenotypes indicated a causal relationship between ADK knockout and NAFLD improvement. To further validate our observation and provide a rationale for the beneficial effects of hepatic ADK knockout on NAFLD, we employed an alternative ADK KO mouse model using the adeno-associated virus tail vein injection strategy.

Our liver-specific homozygous ADK-KO mice generated with Cre-loxp technology died right after the weaning stage, which was similar to the global ADK mutant mouse model; homozygous ADK mutants automatically developed severe steatotic liver and

died within 2 weeks after birth [1]. It is suggested that adenosine is mainly removed by ADA during embryonic development, but ADK takes the place of ADA postnatally. This probably explains the postnatal onset of symptoms of ADK dysfunction in both mice and humans [9]. Since ADK is present throughout the body, mouse models of ADK specifically knocked out in many other tissues are available including pancreatic β-cells [66, 75], endothelial cells [12, 111], and macrophages [83]. Unlike ADK knocked out in the liver, none of the above-mentioned tissues with ADK knocked out are lethal to the animal, suggesting a crucial role of hepatic ADK in survival. Note that our AAV-mediated hepatic ADK-KO mice died 3 weeks after ADK inactivation. In this mouse model, the timing of ADK disruption was manipulated and thus did not allow much room for compensatory or redundancy mechanisms to occur to complicate our observations [129]. This inducible ADK KO mouse model recapitulated the lethal trait of the conditional ADK KO mouse model generated with the Cre-LoxP system and thus confirmed the indispensable role of ADK in living beings.

The weight loss in the TBG virus treated mice could be attributed to increased ketogenesis on one side [130], as we can see the higher levels of ketone bodies in the liver. Ketogenesis is exclusively limited to the liver because the rate-limiting ketogenic enzyme 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) is restricted to the hepatocytes [131]. Ketogenesis usually occurs during prolonged fasting or starvation, and the end products ketone bodies subsequently transport energy from the liver to extrahepatic tissues, the brain in particular [132]. On the other side, lipogenesis was suppressed indicated by the lower levels of the major determinants encoded by gene ACC1 and FAS

[133]. We know CPT1 is a key regulator in fatty acid oxidation [134], which is the preceding process of ketogenesis, so its upregulation was consistent with the higher ketone bodies in the liver. Ketogenesis is good as a short-term energy backup [135]; it is detrimental, however, it is detrimental when it goes rampant because the excessive accumulation of the acidic ketone bodies is toxic to the body[136]. The increased liver weight likely resulted from ketogenic damage [137]. Hypoglycemia has been reported to be a clinical symptom for some of the ADK-deficient patients [8], but the blood glucose levels were not low enough to be lethal to the homozygous ADK KO mice (results not shown). Therefore, the unstrained ketogenesis was likely responsible for the sudden death of the virus-mediated inducible knockout mice 3 weeks after the virus injection, and for the early postnatal mortality of the Cre-LoxP mediated conditional homozygous knockout mice. Liver histology analysis and serum ALT and AST quantification on the TBG virus treated mice should help to provide more insight about this issue in the future.

We reported here that both mouse models indicate a protective nature for ADK inhibition in aspects of diet-induced NAFLD. This was probably ascribed to more lipid oxidation and less lipid synthesis. However, when the fatty acid oxidation was exacerbated to an extent, it was lethal to the affected mice.

CHAPTER V

LIVER-SPECIFIC ADK OVEREXPRESSION INDUCES NAFLD UPON CHOW DIET FEEDING

Introduction

Chronic HFD exposure causes excessive lipid accumulation in the liver, a process leading to fatty liver disease. This is a commonly used animal model for NAFLD. It's uncommon, however, for a mouse model to develop NAFLD without an additional hit [138]. We proved that hepatic ADK levels were responsive to different nutritional challenges, and this response appeared to be of pathogenic importance because ADK deficiency selectively in the liver protected against diet-induced NAFLD.

Since ADK is the principal factor affecting adenosine metabolism, the substrate cycle between adenosine and AMP enables minor changes in ADK function to amplify into major changes in adenosine [139]. Thus, the abnormal activation of ADK with genetic disorders or environmental insults may represent a novel contributor to disease development. This hypothesis has gained support from a study demonstrating that overexpression of ADK *per se* is sufficient to trigger electrographic seizures without insults like brain injury [140]. However, the influences of ADK overexpression on other tissues like in the liver have yet to be discovered.

To directly address the potential contribution of ADK toward the development of the detrimental metabolic consequences of NAFLD, we have engineered liver-specific transgenic overexpression of ADK in mice by introducing an additional transgenic copy

of the full-length ADK gene in the genome (ADK cDNA), which was under the control of the albumin promoter [141]. We aimed to investigate further if liver-specific ADK overexpression would predispose mice fed with a chow diet to NAFLD.

Materials and Methods

Animal studies

Male transgenic ADK overexpression (ADK-TG) mice and gender- and age-matched C57BL/6 wild-type (WT) mice (n=10 per group) were housed, five mice per cage, at 22 ± 1 °C with a 12h: l2h light:dark cycle (lights on at 06:00 am) with free access to water and a standard chow diet. This diet is composed of 24% protein, 18% fat, and 58% carbohydrate of total kcal. Body weight and food intake were monitored weekly. GTT was performed once a month, and body composition detection as well. At the end of seven months, ITT was performed before blood and tissue samples were harvested and stored at -80 °C until further processing as previously described (see Chapter III). All animal experiments and care were approved by the IACUC (Institutional Animal Care and Use Committee) at Texas A&M University (College Station, TX) under National Institutes of Health guidelines.

Confirmation of ADK overexpression in the liver

The hepatic ADK overexpression was confirmed with western blot analysis as described in detail in Chapter III.

Metabolic profiles of hepatic ADK overexpression with regards to aspects of obesity-associated NAFLD

Liver H&E staining and the inflammatory response were accessed in a similar way to Chapter III. Liver tissue Oil Red O staining was performed as described in the literature [142]. Plasma triglyceride concentrations were determined by commercially available kits (BioAssay Systems; Hayward, CA, USA). Lipogenic gene ACC1, FAS, and SREBP-1c expressions were quantified with RT-PCR as detailed in Chapter IV.

EchoMRI

The body weight of each mouse was determined with a digital scale before mice were placed in the EchoMRI 1100 (EchoMRI LLC, Houston, TX, USA) without the use of anesthesia to measure body composition. Parameters including total body fat, lean mass, free water, and total water were measured in a rapid (within a minute) and noninvasive manner. Animal-containing tubes were immediately disinfected with 70% ethanol between each use.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 6.01 (GraphPad Software, Inc., La Jolla, CA, USA). Data were presented as the mean ± SEM. We performed statistical analysis using two-tailed unpaired t-tests. The cutoff for statistical significance was set at a $P<0.05$.

Results

We found that mice with overexpression of ADK in the liver tissues appeared healthy and demonstrated no visible anatomic or behavioral abnormalities. These mice were indistinguishable from the WT mice because they exhibited a similar body weight gain and glucose tolerance (data not shown) with a standard chow diet feeding until 4 months old (Fig. 14A). However, the fat mass diverged as early as 3 months and was significantly higher in ADK-TG mice in contrast to that in the WT mice (Fig. 14C). After that, the body weight and fat mass in the hepatic ADK overexpression mice maintained at a higher level, accompanied with the deterioration of glucose intolerance (data not shown), persisted out to 28 weeks of age. However, food consumption remained the same between the groups (Fig. 14B).

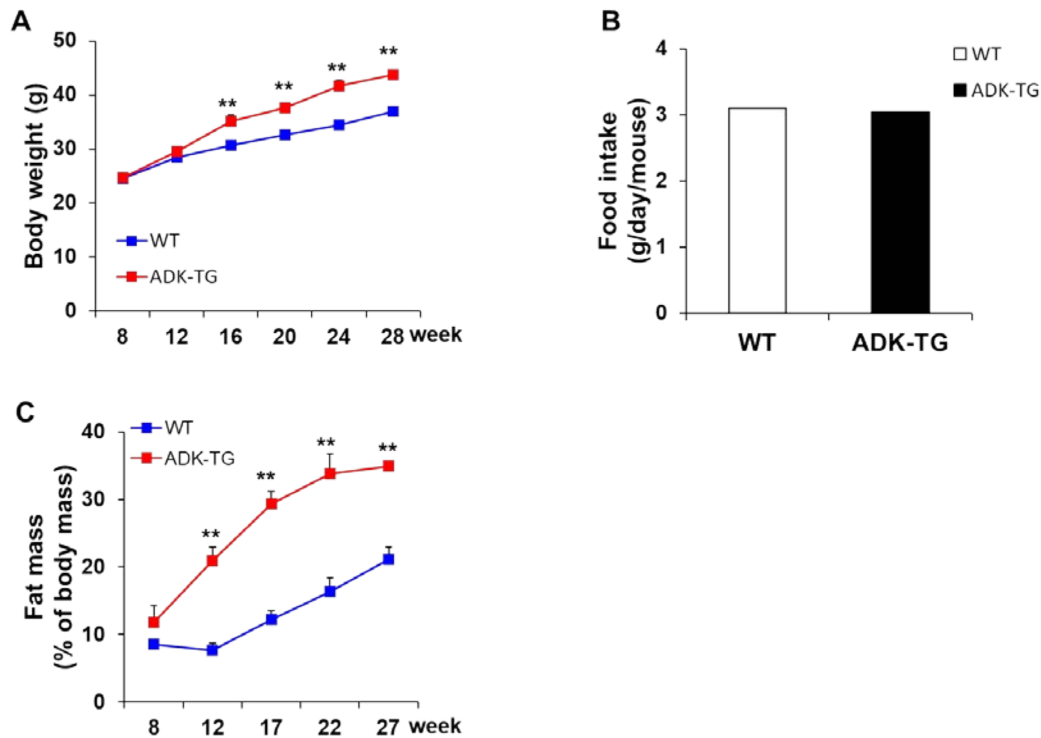

Figure 14: Hepatocyte ADK overexpression promotes weight gain.
This mouse model was generated by placing a full length of ADK cDNA under control of the liver-specific albumin promoter. Male ADK-TG and gender- and age- matched WT mice were fed with a chow diet since birth for 7 months. n= 6-8. (A) Body weight measured weekly. (B) Food intake measured weekly. (C) Fat mass measured with EcoMRI every 4-5 weeks. *, $P<0.05$ and **, $P<0.01$, ADK-TG vs. WT.

Toward the end of the experiment, transgenic mice had higher mean blood glucose levels at any time point than WT mice after the bonus glucose injection with the glucose measured for 2 hours (191 ± 12 vs. 298 ± 32 mg/dl; P = 0.003; Fig. 15A). Accordingly, the ITT data indicated a worse insulin resistance in the transgenic mice. Fifteen minutes after i.p. insulin injection, average blood glucose reduced by only 9% in the ADK-TG mice compared with 32% in the WT mice (Fig. 15B).

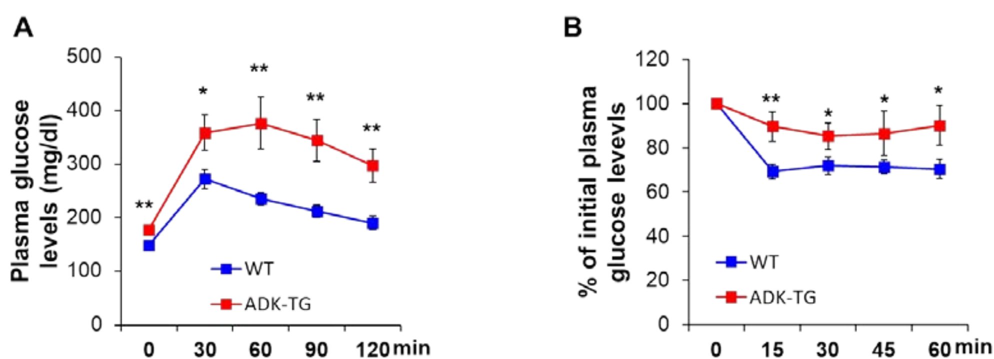

Figure 15: Hepatocyte ADK overexpression induces glucose intolerance and insulin resistance.
This mouse model was generated by placing a full length of ADK cDNA under control of the liver-specific albumin promoter. Male ADK-TG and gender- and age-matched WT mice were fed with chow diet since birth for 7 months. n=6-8. Data were presented as the mean ± SEM, n= 6-8. (A) Glucose tolerance test (GTT). (B) Insulin tolerance test (ITT). For GTT and ITT, mice fasted for 4 h and were injected with a single dose of D-glucose (2 g/kg BW, 0.1 mL/10g BW) or insulin (1 U/kg BW, 0.1 mL/10g BW), respectively, and plasma glucose levels were determined at the indicated time points. *, P<0.05 and **, P<0.01, ADK-TG vs. WT.

Our transgenic mice showed a moderate hepatic ADK overexpression (Fig. 16A). The liver was slightly but significantly heavier in these mice (Fig. 16B). Also, hepatic ADK overexpression promoted fat deposition in the liver, as assessed using H&E and Oil Red O staining (Fig. 16C). To address the possible molecular mechanisms of the excessive ectopic fat accumulation in ADK-TG mice, mRNAs encoding key enzymes of hepatic lipid metabolism were quantified by RT-PCR. On a chow diet, ADK-TG mice had significantly upregulated expressions of the mRNAs encoding the rate-limiting enzymes of lipogenesis ACC1 and FAS (Fig. 16D), suggesting an increased lipid synthesis in the liver. Given the elevated ACC1 and FAS expression, it is not surprising that the mRNA for the major transcriptional regulator of the lipogenic pathways, sterol

response element binding protein (SREBP) 1c, was also induced in ADK-TG mice (Fig. 16D). The circulating triglyceride levels were significantly higher in the transgenic mice as well (Fig. 16E), which was in agreement with the higher lipid synthesis in the liver.

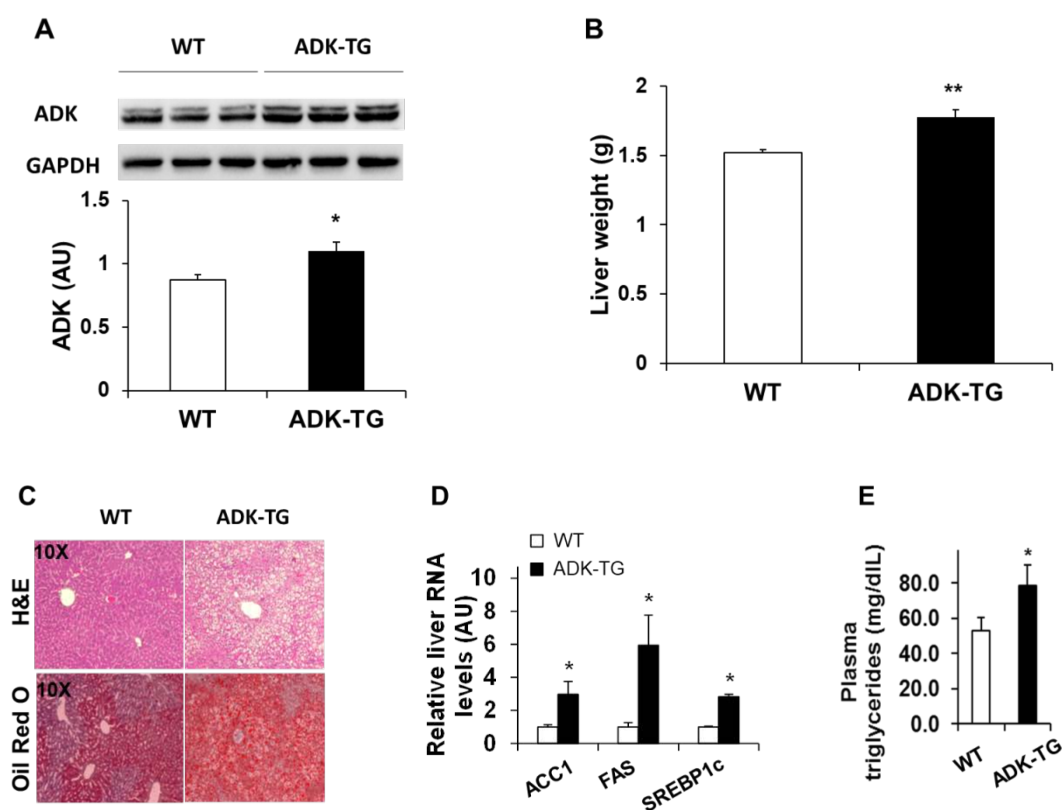

Figure 16: Hepatocyte ADK overexpression promotes fat deposition in the liver. This mouse model was generated by placing a full length of ADK cDNA under control of the liver-specific albumin promoter. Male ADK-TG and gender- and age-matched WT mice were fed with chow diet since birth for 7 months. n=6-8. Data are presented as the mean ± SEM, n= 6-8. (A) Hepatic ADK levels determined by western blot analysis and quantified with densitometry. (AU, arbitrary unit). (B) Liver weight. (C) Liver histology: H&E staining and Oil Red O staining. (D) Liver mRNA levels of key lipid metabolic enzymes (genes) were analyzed using real-time PCR. (E) Plasma triglyceride levels. *, P<0.05 and **, P<0.01, ADK-TG vs. WT.

Lastly, we examined the inflammatory makers in the liver tissues of these mice. Both the phosphorylation of p46 and p65, the subunit of JNK and NFκB, respectively, increased in the ADK-TG mice compared to the WT mice (Fig. 17), suggesting an enhanced pro-inflammatory response in the transgenic mice.

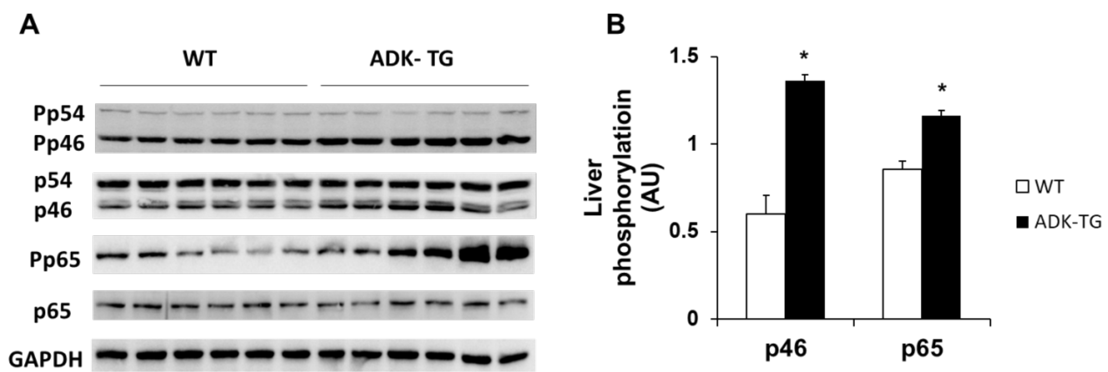

Figure 17: Hepatocyte ADK overexpression exacerbates hepatic inflammation. This mouse model was generated by placing a full length of ADK cDNA under control of the liver-specific albumin promoter. Male ADK-TG and gender- and age-matched WT mice were fed with a chow diet since birth for 7 months. n=6-8. (A) Liver inflammatory responses indicated by western blot analysis. (B) Quantification of western blots with Image J using densitometry and results normalized to GAPDH. (AU, arbitrary unit). *, P<0.05 and **, P<0.01, TBG virus vs. Ctrl virus.

Taken together, mice with hepatic ADK overexpression displayed accelerated weight gain, more far mass, exacerbated glucose intolerance and insulin resistance, and robust dyslipidemia and fatty liver, as well as enhanced hepatic inflammation upon a standard chow diet feeding.

Discussion

Here, we reported that hepatic ADK overexpression *per se* rendered mice more susceptible to NAFLD even without dietary insults like an HFD challenge. These transgenic mice appeared normal at a younger age, but their metabolic profiles deteriorated rapidly since 4 months old, perhaps earlier than that, because an increased fat mass/body weight ratio was observed at 3 months old. Clues are limited at this point concerning the exact mechanisms related, whereas aging is likely an inducer since it shares similar cellular pathways as obesity [143]. Notably, dissecting and weighing of fat depots in individual mice can also be used to measure the total fat mass as we did for studies in the previous chapters. However, this method is less accurate due to the challenge to completely dissect the fat portion integrated in the organs or tissues. Thanks to the application of EcoMRI, we can analyze the body composition more accurately. EchoMRI was not performed in animals in previous chapters as the instrument was purchased and installed after those mice were harvested.

The concentration of adenosine is physiologically maintained primarily by ADK at a low level with a narrow range of 30-200nM [19]. Adenosine is released locally and rapidly in the setting of metabolic stress such as hypoxia and ischemia to prevent damage to the cells or organs [5, 144]. High levels of adenosine were reported to protect the brain from cell injury, which justifies the application of adenosine argumentation for anti-epilepsy in the preclinical studies [145, 146]. In this study, the hepatic ADK levels were genetically manipulated to be modest overexpression, which could dramatically reduce adenosine concentration because slight changes in ADK activity could be translated to

huge changes in adenosine levels due to the substrate circle between adenosine and AMP [139]. Remarkably, adenosine is a substrate inhibitor of ADK [10], therefore, the adenosine lowering process might be further amplified due to the diminishing suppression of adenosine on ADK. Since the cell-type-specific changes of ADK expression coincide with major changes of aspects of NAFLD, our observations strongly implicate hepatic ADK overexpression as a major contributing factor in the process that turns a normal liver into a fatty liver.

Developing and utilizing an informative animal model to recapitulate the pathophysiology of diseases being investigated has continued to be an unresolved issue faced in many fields. Although many dietary and genetic animal models of NAFLD have been developed so far [147], which has provided valuable guidance in understanding the pathogenesis and progression of NAFLD, our hepatic ADK overexpression mouse model stands out in the aspect that it spontaneously develops hepatic inflammation without a secondary insult, such as an HFD feeding or an MCD diet feeding [148]. However, more investigations are warranted to further confirm if this hepatic ADK overexpression mouse model recapitulates all characteristics of human disease.

CHAPTER VI

MECHANISMS UNDERLYING ADK ACTIONS: $A_{2A}R$ AND DNA METHYLATION.

Introduction

Our animal studies with liver-specific ADK knockout or overexpression mouse models support a causal effect of ADK dysfunction in the pathogenesis of NAFLD. Briefly, hepatic ADK deficiency rescued the HFD-induced NAFLD, and hepatic ADK overexpression contributed to the development of NAFLD even without a dietary insult. However, the mechanisms underlying the ADK actions remain obscure.

The fluctuation of ADK function would directly, rapidly and tremendously affect the adenosine homeostasis, which is at the crossroad of adenosine signaling and transmethylation reactions [10]. On one hand, adenosine signals through its four GPCRs A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R to increase or decrease cAMP, stimulate the release of calcium ions, and activate MAPK pathways, including ERK1/ERK2, JNK and p38 MAPK [149], which ultimately contributes to the regulation of various biological processes. Out of the 4 adenosine receptors, $A_{2A}R$ plays an important role in the regulation of inflammatory and immune responses [150]. The previous work of ours and others suggest a protective role for $A_{2A}R$ in HFD-induced NAFLD [151, 152], therefore we speculated that the $A_{2A}R$ signaling was necessary for the beneficial effects of ADK deficiency on NAFLD. On the other hand, ADK is an obligatory end product of the methylation reactions. DNA methylation involves the transfer of a methyl group to the

C5 position of the cytosine to form 5-methylcytosine (5mC). The methylation of CpG islands results in stable silencing of gene expression [153]. When ADK dysfunction, for example ADK deficiency, adenosine would be accumulated and drive the reversible reaction between SAH hydrolysis and SAH synthesis to SAH synthesis and generate more SAH. SAH is an inhibitor of DNMT and thus suppresses DNA methylation of target genes, which leads to more transcription and expression of those genes. Emerging evidence suggests that aberrant DNA methylation patterns may indicate an increased susceptibility for NAFLD [154]. Could DNA methylation-related epigenetic reprogramming also contribute to the actions of ADK?

To directly address the role of $A_{2A}R$ in this regard, we generated hepatic $A_{2A}R$ knockout alone and hepatic $A_{2A}R$ and ADK double knockout (DKO) mouse models. We aimed to see if $A_{2A}R$ is necessary for ADK deficiency's beneficial effects on aspects of NAFLD upon HFD feeding. Moreover, the global DNA methylation of liver tissues from hepatic ADK knockout or overexpression mice from previous studies was determined to assess the role of DNA methylation with regards to ADK action in NAFLD.

Materials and Methods

Mouse generation and breeding

Hepatic $A_{2A}R$ knockout mice were generated similarly as the ADK knockout mice by crossing $A_{2A}R^{F/F}$ mice with Alb-cre mice. $A_{2A}R$ and ADK double knockout mice were created by mating AlbCre-$ADK^{F/+}$ mice with AlbCre-$A_{2A}R^{F/F}$ mice. Notably,

homozygous liver-specific ADK KO mice cannot survive; the ADK was not completely knocked out in the DKO mice. After several rounds of breeding, we got enough $A_{2A}R$ knockout alone mice (AlbCre^{+}-ADK$^{+/+}$ $A_{2A}R^{F/F}$) and DKO mice (AlbCre^{+}-ADK$^{F/+}$ $A_{2A}R^{F/F}$). All mice were provided with water ad libitum and maintained on a 12:12-hour light:dark cycle in a specific pathogen-free facility at 22 ± 1 °C. All animal experiments and care were approved by the IACUC (Institutional Animal Care and Use Committee) at Texas A&M University (College Station, TX) per National Institutes of Health guidelines.

Confirmation of the liver-specific DKO mouse model.

The genotype of liver-specific DKO and $A_{2A}R$ knockout alone ($A_{2A}R$ KO) were identified by PCR-based genotyping performed on tail-extracted genomic DNA at weaning and were retrospectively confirmed with western blot analysis in experimental animals.

Animal studies

Five-to-six-week old male liver-specific DKO and $A_{2A}R$ KO mice (n=6-8 per group) were fed with an HFD for 12 weeks. During the 12-week feeding period, body weight and food intake were monitored weekly. GTT and ITT were performed in the same way as in Chapter III. After the feeding regimen, mice were anesthetized, blood samples were collected and plasma obtained by centrifugation at 3000rpm for 15 min at 4°C. Tissue samples were removed, weighed and snap-frozen immediately in liquid nitrogen and then stored in -80 °C for further analysis.

EchoMRI

The body weight of each mouse was determined with a digital scale before mice were placed in the EchoMRI 1100 (EchoMRI LLC, Houston, TX, USA) to measure body composition without the use of anesthesia. Parameters including total body fat, lean mass, free water, and total water were measured in a rapid (within a minute) and noninvasive manner. Animal-containing tubes were immediately disinfected with 70% ethanol between each use.

Hepatic inflammation detection

The inflammatory markers pNFκB/NFκB and pJNK/JNK in the liver tissues were determined with western blot assays as described in Chapter III.

Cell culture and treatment

Primary hepatocytes were isolated from chow-diet-fed male A_{2A}R KO and DKO mice at 10 weeks of age as previously described [155]. After attachment, hepatocytes were incubated in M199 supplemented with 10% FBS and 100 U/ml penicillin and 100 μg/ml streptomycin for 24 hr. Cells were then treated with palmitate (Pal, 250 μM, conjugated in bovine serum albumin (BSA)) or BSA for 24 hr, and stained with Oil Red O for 1 hr.

Liver immunohistochemistry for DNA methylation

The formalin-fixed and paraffin-embedded liver tissue sections were deparaffinized and rehydrated followed by being boiled in Target Retrieval Solution (Dako, TRS, CA, USA). After washed with PBS, these sections were blocked with 2% BSA for 1 hour and then incubated with primary antibodies against 5-methylcytosine (5mC) overnight at 4

°C. Thereafter, the samples were incubated with secondary antibodies (Life Technologies, Inc., Carlsbad, CA, USA), followed by incubation of peroxidase-conjugated streptavidin, with multiple washes between each incubation. DAB (3,3' diaminobenzidine) chromogen was applied to develop the slides. Images were taken under a standard upright microscope with a digital camera.

Microarray analysis of gene expression patterns in mouse livers

Liver tissues of male homozygous ADK knockout (AlbCre^{+}-ADK$^{F/F}$) and control (AlbCre^{+}-ADK$^{+/+}$) mice (n=3) were harvested at 3 weeks of age. Total RNA was isolated according to the manufacturer's instructions. Equal amounts of liver RNA samples from mice of the same group were pooled together and split into duplicate samples for cDNA synthesis and application to the microarrays (Affymetrix, Santa Clara, CA). Data were analyzed with the Affymetrix GeneChip Expression Analysis Software and differential expression of selective liver mRNAs were expressed as AlbCre^{+}-ADK$^{F/F}$ mice to AlbCre^{+}-ADK$^{+/+}$ mice ratios in table 1.

Statistical analysis

Statistical analyses were performed with GraphPad Prism 6.01 (GraphPad Software, Inc., La Jolla, CA, USA). Data are presented as mean ± SEM. Two-tailed unpaired t-tests were used for comparisons between the two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

There was no significant difference in body weight throughout HFD feeding between the two groups (Fig. 18A). The fat mass in DKO mice was comparable to that in the A_{2A}R KO mice (Fig. 18B). However, the plasma glucose levels in the DKO mice

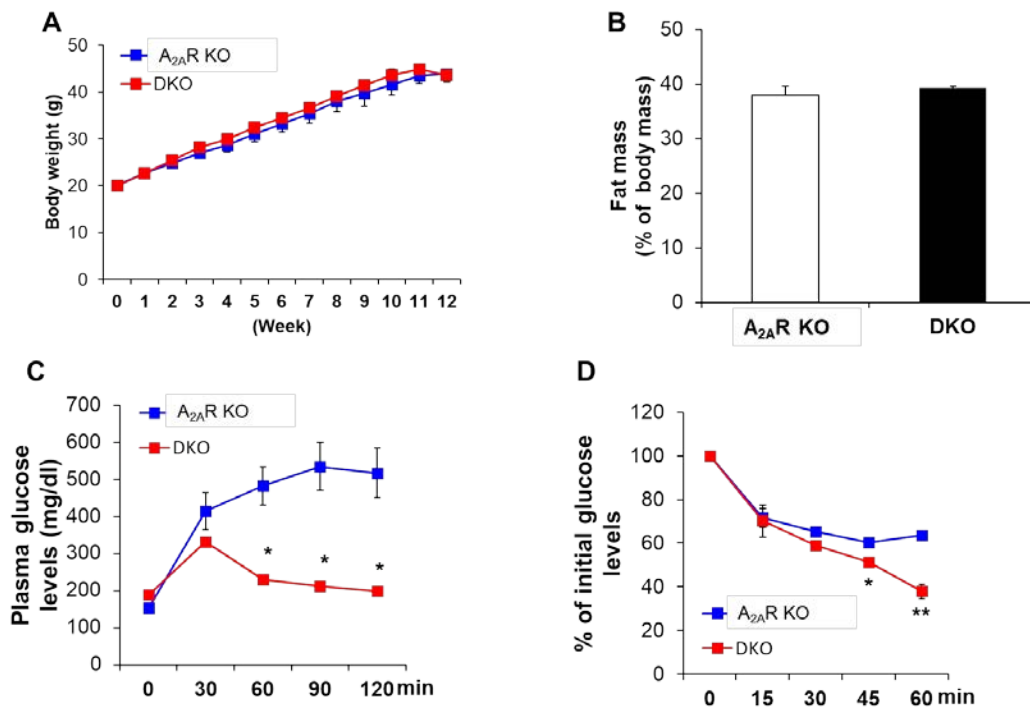

Figure 18: DKO protects against glucose intolerance and insulin resistance. This mouse model was generated by created by crossing AlbCre-$ADK^{F/+}$ mice with AlbCre-$A_{2A}R^{F/F}$ mice. Five-to-six-week old male liver-specific DKO and A_{2A}R KO mice were fed with an HFD for 12 weeks. Data are presented as mean±SEM, n= 6-8. (A) Body weight. (B) Fat mass measured with EchoMRI. (C) Glucose tolerance test (GTT). (D) Insulin tolerance test (ITT). For GTT and ITT, mice fasted for 4 h and were injected with a single dose of D-glucose (2 g/kg BW, 0.1 mL/10g BW) or insulin (1 U/kg BW, 0.1 mL/10g BW), respectively, and plasma glucose levels were determined at the indicated time points. *, $P<0.05$ and **, $P<0.01$, DKO vs. A_{2A}R KO.

during the time course of GTT remained higher in comparison to $A_{2A}R$ KO mice (Fig. 18C). Similarly, the glucose levels dramatically decreased in the DKO mice upon an intraperitoneal administration of an insulin bonus, in contrast to the less glucose-lowering effects of insulin in the $A_{2A}R$ KO mice (Fig. 18D).

In the livers of DKO mice, inflammation response was dampened by about 20% in terms of the phosphorylation status of subunits of JNK and NFκB (Fig. 19).

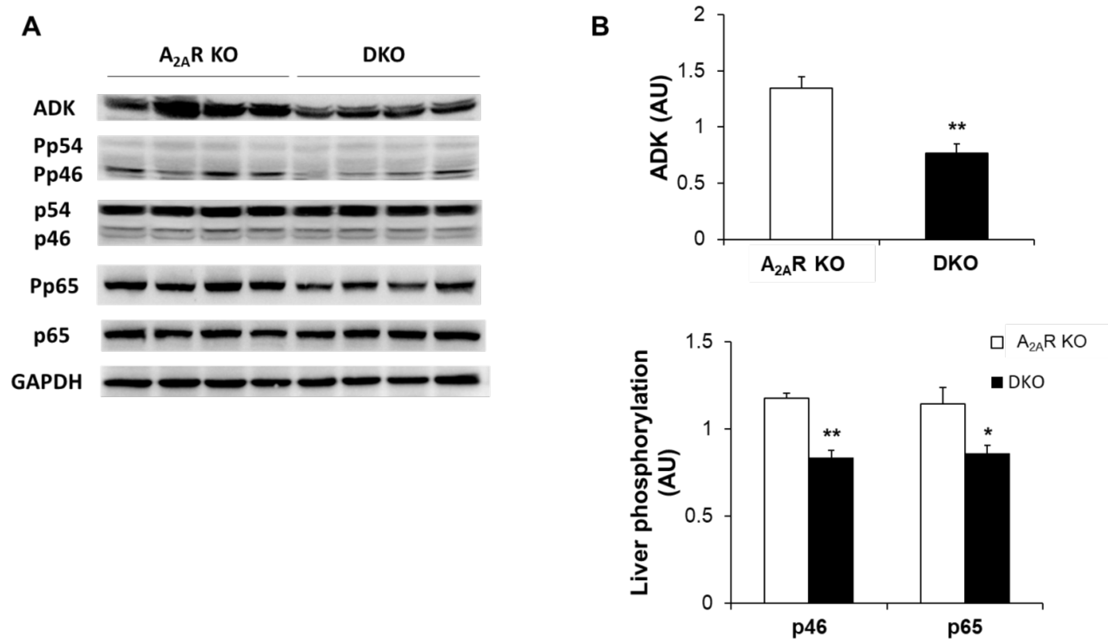

Figure 19: DKO attenuates hepatic inflammation.
This mouse model was generated by created by crossing AlbCre-$ADK^{F/+}$ mice with AlbCre-$A_{2A}R^{F/F}$ mice. Five-to-six-week old male liver-specific DKO and $A_{2A}R$ KO mice were fed with an HFD for 12 weeks. Data are presented as mean ± SEM, n= 6-8. (A) Liver inflammatory responses indicated by western blot analysis. (B) Quantification of western blots with Image J using densitometry and normalized to GAPDH. (AU, arbitrary unit). *, $P<0.05$ and **, $P<0.01$, DKO vs. $A_{2A}R$ KO.

In agreement with the observations *in vivo*, we found that fat deposition was less prominent in primary hepatocytes isolated from DKO mice in comparison with $A_{2A}R$ KO mice during basal conditions (Fig. 20). Upon palmitate challenge, the fat accumulation in hepatocytes of DKO mice increased visibly but to a much lesser extent compared to $A_{2A}R$ KO mice (Fig. 20).

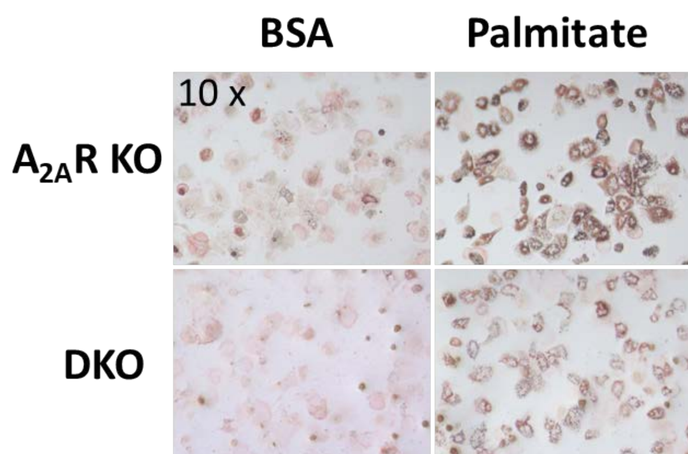

Figure 20: DKO attenuates hepatocyte fat deposition induced by palmitate in vitro. Primary hepatocytes were isolated from chow-diet-fed male $A_{2A}R$ KO and DKO mice at 10 weeks of age. After attachment, hepatocytes were treated with palmitate (Pal, 250 μM) or BSA for 24 hr, and hepatocyte fat deposition was assessed with Oil Red O staining.

With regards to DNA methylation, the 5mC staining of livers sections of ADK-TG mice indicated a global DNA hypermethylation, and of TBG virus-mediated ADK KO mice a global DNA hypomethylation (Fig. 21).

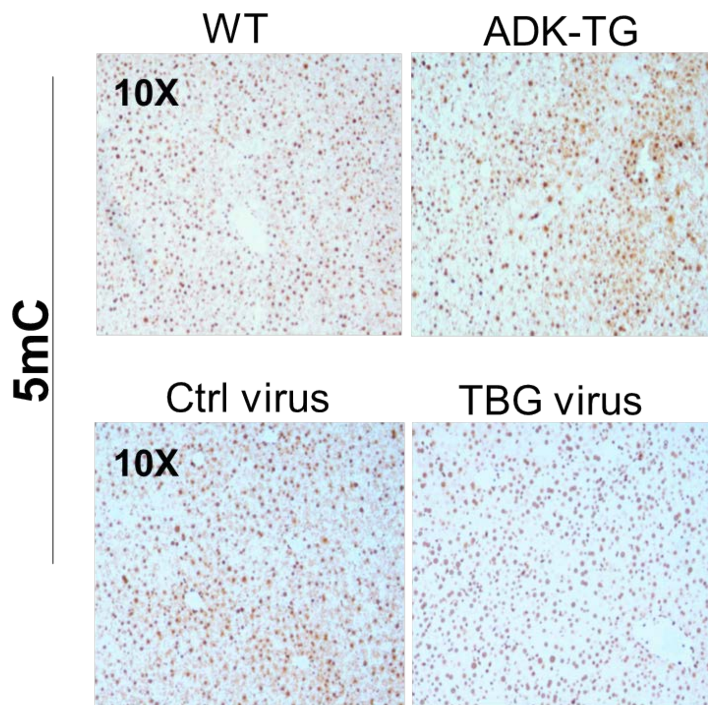

Figure 21: ADK and DNA methylation.
Liver global methylation status from ADK-TG and TBG virus mice indicated by 5mC methylation staining with DAB detection of antibody (brown).

To further investigate the gene expression patterns in the liver tissues of ADK-deficient mice, we performed microarray analysis with liver tissues from homozygous ADK knockout and control mice. We observed a significant increase in peroxisome

proliferator-activated receptor (PPARα), CPT1, and $A_{2A}R$ mRNA levels in ADK-deficient mice (Table 1). Besides the genes aforementioned, several other genes were observed to be affected by ADK deficiency (Table 1). All these genes selected are involved in either lipid metabolism or AMP metabolism.

Table 1: Expression of selected liver genes altered by ADK deficiency.

Gene symbol	Gene name	Fold of change	*P* value
Crebbp	**CREB binding protein**	**5.06**	**9.98E-06**
Adora2a	**adenosine A2a receptor**	**4.02**	**0.001833**
Ampd1	**adenosine monophosphate deaminase 1**	**3.10**	**0.000662**
Ak1	**adenylate kinase 1**	**5.32**	**1.49E-06**
Fasn	**fatty acid synthase**	**0.25**	**6.06E-15**
Cdk8	**cyclin-dependent kinase 8**	**2.69**	**1.35E-08**
Cpt1a	**carnitine palmitoyltransferase 1a**	**10.54**	**9.48E-25**
Ppara	**peroxisome proliferator activated receptor alpha**	**2.32**	**2.95E-05**
Bdh2	**3-hydroxybutyrate dehydrogenase, type 2**	**11.29**	**1.21E-11**

Discussion

Given that adenosine pools inside and outside of cells are in a dynamic exchange via ENTs, the extracellular adenosine signaling is supposed to be altered following the changes in intracellular ADK activity. Cao et al. proposed that the ADK inhibitor ABT-702 could suppress cisplatin-induced nephrotoxicity via enhanced A_1R and $A_{2B}R$ signaling [156]. For the current project, we first assessed whether enhanced adenosine signaling was responsible for the protective effects of ADK deficiency. Our observations of the DKO mouse study suggested that ADK deficiency was still protective for HFD-induced NAFLD without the presence of $A_{2A}R$ signaling as supported by the improved glucose intolerance and insulin resistance, and reduced hepatic inflammation in the DKO mice. However, these effects had nothing to do with weight loss for the comparable body weight and fat mass between the two groups. That is, the $A_{2A}R$ signaling played a limited, if not non-existent, role in the actions of ADK inhibition in improving metabolic profiles of the mouse NAFLD model. This speculation had been partly confirmed with the primary hepatocyte cell study. An adenosine receptor-independent mechanism for ADK actions is yet to be discovered.

Therefore, we further explored if the DNA methylation related mechanisms were the major mediators of ADK actions. DNA methylation is an epigenetic process during which 5mC is formed by adding a methyl group onto the 5-carbon of the cytosine ring with the help of DNA methyltransferase (DNMT) [157]. 5mC is ideal for distinguishing the unmodified cytosine base from the methylated cytosine base for DNA methylation research [158]. We observed a global DNA hypermethylation in liver tissues of hepatic

ADK overexpression mice and hypomethylation in KO mice, corresponding to exacerbated and ameliorated NAFLD, respectively. Our observations seem to be contrary to the general tendency of hypothmethylation of hepatic DNA found in the NAFLD patients [159], or the proof-of-concept that reduced DNA methylation is correlated to higher susceptibility to NASH in mice [160]. We have very limited information to reference to provide a convincing rationale for this discrepancy, but epigenetic programming is subject to the influence of so many factors, notable differences in the species, diets, and measurements could possibly account for the variability. It is noteworthy that similar ADK and DNA methylation patterns have been reported in the study conducted by Boison et al. showing that the astrocyte-specific ADK overexpression in epilepsy correlated with DNA hypermethylation, and reduced ADK expression induced a lowering global DNA methylation [145].

Moreover, it is of great importance to pinpoint the methylation and subsequent expression of which specific genes are influenced. We saw the expression of a bunch of genes altered through the Microarray analysis. Considering the established paradigm that increased lipogenesis critically contributes to the development of NAFLD, we took a closer look at those genes highly related to lipid metabolism. Remarkably, we observed significant increase in PPARα and CPT1 in liver tissues of homozygous ADK KO mice compared to ADK intact mice. PPARα is a transcription factor highly expressed in hepatocytes with many target genes involved in hepatic fatty acid oxidation and ketogenesis, such as CPT1[161]. The high level of PPARα expression could explain the higher levels of ketone bodies in the livers of AAV-mediated ADK KO mice. Besides,

PPARα has been proved to be anti-inflammatory through inhibiting the NF-κB pathway [162]. Given the co-existence of these actions of PPARα, it is possible that the higher PPARα resulted from DNA hypomethylation in hepatic ADK-disrupted mice acts through promoting fatty acid oxidation to improve hepatic steatosis. Alternatively, higher PPARα might act through two parallel pathways to facilitate fatty acid oxidation and suppress hepatic inflammatory responses.

Strikingly and interestingly, the $A_{2A}R$ mRNA was upregulated in the ADK-deficient mice. Our previous work has confirmed that $A_{2A}R$ activation protects against diet-induced NAFLD through its duel functions, suppressing inflammation and inhibiting fatty acid synthesis [151]. Given the beneficial effects of ADK deficiency on top of $A_{2A}R$ absence, we speculate that $A_{2A}R$ does contribute to but does not assume a predominant role in mediating the actions of ADK.

Note that two isoforms of ADK exist in the mammals, with the long isoform denoted ADK-L, and the short isoform ADK-S. It is proposed that ADK-L is located in the nucleus and proposed to be primarily responsible for maintaining the intracellular transmethylation reactions, while the ADK-S is located in the cytoplasm and largely determines the extracellular adenosine signaling [10]. However, in the current study, we did not try to differentiate the two isoforms and thus failed to provide more information in this aspect, which warrants further investigation.

CHAPTER VII

CONCLUSIONS

Even though the discouraging toxicological data for the ADK-based clinical candidates have hindered the basic and clinical investigations for decades [88], interest in ADK inhibition has been fueled by emerging preclinical data in the areas of neurology, cardioprotection, diabetics, etc. Our animal studies with liver-specific ADK knockout or overexpression mouse models support a causal effect of ADK overexpression in the pathogenesis of NAFLD. Briefly, hepatic ADK deficiency rescued the HFD-induced NAFLD, but hepatic ADK overexpression contributed to the development of NAFLD even without a dietary insult. Mechanistically, ADK overexpression interfered with the transmethylation reactions and the subsequent DNA hypomethylation of target genes, PPARα in particular, which contributed to the pathological process of NAFLD, and in turn was slightly enhanced by the reduction of the $A_{2A}R$ signaling. Taken into possible unknown mechanisms, the actions of ADK in NAFLD may be multilayered. Nevertheless, to the best of our knowledge, this is the first study to demonstrate a robust cause-and-effect relationship between ADK dysfunction and NAFLD.

However, we acknowledge limitations to our project. In terms of adenosine signaling, the other adenosine receptors have not been involved in the current study. Therefore, further investigation with non-selective adenosine receptor antagonists or the quad-knockout mouse model, which lacks all four adenosine receptors, should be conducted [163]. Moreover, PPARα methylation status has to be determined. Bisulfite

sequencing is a commonly used method in this regard. Third, more intervention experiments with cell cultures have to be performed either to recapitulate the *in vivo* phenotypes or to confirm the mechanisms *in vitro*. At the time being, we have some technique issues in respect to primary hepatocyte isolation: 1) Cre-Loxp KO mice died too early to get enough primary hepatocytes isolated; 2) the phenotypes of AAV mediated KO mice were time-sensitive, so a more accurate time point within the window of week 2 to week 3 has to be determined for isolation; 3) ADK-TG mice had huge amount of lipids which badly affected hepatocytes isolation and cell viability. Last but not least, it would be interesting to further explore the role of ADK in methionine-choline deficient (MCD) diet-induced nonalcoholic steatohepatitis (NASH) model, which is an advanced stage of NAFLD.

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